Role of Twist1 in metabolism of repeatedly stimulated Th1 cells

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List of Abbreviations

a.a.	after activation
APCs	antigen presenting cells
Blimp1	B lymphocyte-induced maturation protein 1
bHLH	basic Helix-loop-helix
BM	bone marrow
BreA	BrefeldinA
BSA	bovine serum albumin
CAT	catalase
CD	cluster of differentiation
Ct	threshold cycle
DAPI	4′,6-Diamidin-2-phenylindol
dNTPs	desoxynucleosidtriphosphate
FACS	fluorescence activated cell sorting
FAS	Fatty acid synthesis
FCS	fetal calf serum
FITC	Fluoresceinisothiocyanat
FSC	forward scatter
GPX	Glutathionine peroxidase
G6P	Glucose-6-phosphate
GVHD	Graft versus host disease
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IFNγ	Interferon γ
IL-	Interleukin-
IL-12Rβ2	IL-12 receptor beta 2 subunit
KO	knockout
LN	lymph node
MACS	magnetic activated cell sorting
MHC	Major Histocompatibility Complex
mi RNA	microRNA
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor of *kappa* light polypeptide gene enhancer in B-cells
OVA	Ovalbumin
OXPHOS	Oxidative phosphorylation
PMA/Iono	PMA/Ionomycin
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PE	Phycoerythrin
PKA	Protein kinase A
PKB/Akt	Protein kinase B/Akt
PKC	Protein kinase C
PMA	phorpolmyristic acid
pol	polymerase
PPAR	Peroxisome proliferator-activated receptor
PP2	Protein phosphatase 2
qRT	quantitative real-time
RNA	ribonucleic acid
RA	rheumatoid arthritis
Rpm	rounds per minute
RPMI	Roswell Park Memorial Medium
RT	reverse transcription
siRNA/shRNA	small inhibitory RNA/short hairpin RNA
SNP	single nucleotide polymorphism
SOD	Superoxide mutase
STAT	signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TCA	tricarboxylic acid cycle
TCR	T cell receptor
TGFβ	Tumor growth factor β
Th	T helper
TNFα/ TNFαR	Tumor necrosis factor α/TNFα receptor
Treg	regulatory T cell
41BB	TNFR superfamily member 9 or CD137

Summary

The treatment of human arthritis and murine model of colitis with depleting CD4 antibodies leads to the significant amelioration of an established disease, arguing that CD4+T lymphocytes are a driving force and a relevant therapeutic target in chronic inflammatory diseases (Horneff et al. 1991; Brett et al. 1996; Emmrich et al. ; Brasseit et al. 2016). Nevertheless, the vast majority of CD4+ T lymphocytes residing in the inflamed tissue itself are neither proliferating, nor do they express effector cytokines (Firestein and Zvaifler 2002), except Interferon-γ. How do CD4+ T cells adapt their metabolism for survival in the inflamed tissue? In contrast to the activated T cells, memory T cells are more dependent on fatty acid oxidation for their maintenance (van der Windt and Pearce 2012; van der Windt et al. 2012; Buck et al. 2015; Pearce et al. 2009). At the same time, memory CD8 T cells synthesize fatty acids intracellularly from imported glucose (Sheriff et al. 1995; O'Sullivan et al. 2014), a mechanism to gain energy for cell longevity (Cui et al. 2015). This thesis describes analysis of the metabolism of CD4 T lymphocytes driving chronic inflammation and persisting at the site of inflammation, exemplified by cells that reside in the inflamed tissue of patients with the rheumatic disease juvenile idiopathic arthritis. To specifically take aim at the CD4+ T lymphocytes persisting at the site of inflammation, it is important to determine how these cells adapt their metabolism. We show that pathogenic CD4+ CD45RO+ PD1+ CXCR5- T cells that were isolated from the synovial fluid of patients with juvenile idiopathic arthritis are dependent on a fatty acid oxidation for survival *ex vivo*. Their survival can be blocked by blocking FAO with Etomoxir, pointing to the option of targeting such cells by metabolic interference. Furthermore, CD4+ CD45RO+ PD1+ CXCR5- T cells had upregulated expression of Twist1, a hallmark transcription factor of T lymphocytes persisting in the inflamed tissues of patients with chronic-inflammatory diseases of joints or the gut (Niesner et al. 2008). Expression of Twist1 is specific for Th1 lymphocytes which have repeatedly been re-stimulated *in vitro*, or isolated from inflamed tissue (Niesner et al. 2008). This transcription factor dampens immunopathology caused by the T cells (Niesner et al. 2008), and supports their persistence, by inducing microRNA148a, which regulates expression of the proapoptotic protein Bim (Haftmann et al. 2015). Additionally, there is already evidence that Twist1 regulates metabolic processes. In myocytes Twist1 regulates glycogen storage and inflammation (Mudry et al. 2015a). In adipocytes, Twist1 stimulates fatty acid oxidation by inducing expression of *Carnitine palmitoyltransferase I* (CPT1) mRNA, a key player of FAO (Pettersson et al. 2010; Dobrian 2012). This thesis shows, through conditional genetic

inactivation of Twist1 in CD4+ T lymphocytes, that Twist1 also regulates the metabolism of CD4 T lymphocytes of chronic inflammation, by downregulating glycolysis, promoting fatty acid synthesis and protecting the cells from ROS. Twist1 does so by downregulation of *Myc* and upregulation of the genes involved in mitochondrial biogenesis and fatty acid synthesis such as *Atf5, Bnip3, Gpt2, Gpx4, Myc, Oxct1, Rdh11, Slc2a1, Slc2a3, Paics, Pfkp,* and *Ppp1r3b*. Additionally we show that Twist1 deficient repeatedly reactivated murine Th1 cells are unable to survive on fatty acid oxidation and have increased levels of lipid peroxidation.

Zusammenfassung

CD4+ T Lymphozyten spielen eine entscheidende Rolle bei chronisch entzündlichen Erkrankungen und stellen deshalb relevante therapeutische Ziele dar. So führt die Behandlung mit Antikörpern, die CD4+ Zellen depletieren, nicht nur zur Verbesserung der Symptome im murinen Colitis-Modell, sondern auch bei Patienten mit rheumatoider Arthritis (Horneff et al. 1991; Brett et al. 1996; Choy et al. ; Emmrich et al. ; Brasseit et al. 2016).

Ein Großteil der CD4+ T Lympphozyten befindet sich direkt im entzündeten Gewebe, wobei die Zellen mit Ausnahme von Interferon-γ weder Effektorzytokine ausschütten, noch proliferieren (Firestein and Zvaifler 2002). Es stellt sich die Frage, wie diese Zellen im entzündeten Gewebe überleben können und wie sie ihren Stoffwechsel an die dortigen Bedingungen anpassen. Im Gegensatz zu aktivierten T Zellen, basiert der Metabolismus von Gedächtnis T Zellen auf der Oxidation von Fettsäuren (van der Windt and Pearce 2012; van der Windt et al. 2012; Buck et al. 2015; Pearce et al. 2009). Die Fettsäuren werden dabei unter anderem intrazellulär aus Glucose synthetisiert, die zuvor aufgenommen wurde (Sheriff et al. 1995; O'Sullivan et al. 2014). Dieser Mechanismus ist gleichzeitig notwendig und ausreichend für die Langlebigkeit von CD8 T Zellen (Cui et al. 2015).

Diese Arbeit beschreibt am Beispiel der Juvenilen Idiopathischen Arthritis (JIA) die Analyse des Stoffwechsels von CD4+ T Lymphozyten, die chronische Entzündungen antreiben und im entzündeten Gewebe lange Zeit persistieren. Pathogene CD4+ CD45RO+ PD1+ CXCR5- T Zellen wurden hierfür aus Synovialflüssigkeit von Patienten mit JIA isoliert und gezeigt, dass auch bei diesen Zellen der Stoffwechsel auf Fettsäureoxidation beruht. Wurde die Fettsäureoxidation durch Etomoxir blockiert, starben die Zellen. Die Störung des Stoffwechsels dieser Zellen könnte somit eine Option für einen neuen Therapieansatz darstellen. Zusätzlich war die Expression des Transkriptionsfaktors Twist 1 in diesen CD4+ CD45RO+ PD1+ CXCR5- T Zellen hochreguliert. Twist 1 ist ein Marker für T Lymphozyten, die in entzündetem Gewebe von Patienten mit chronisch-entzündlichen Erkrankungen der Gelenke oder des Darmes persistieren (Niesner et al. 2008). Untersuchung *in vitro* ergaben außerdem, dass Twist 1 spezifisch von Th1 Lymphozyten exprimiert wird, die mehrfach re-stimuliert wurden (Niesner et al. 2008). Dieser Transkriptionsfaktor wirkt einerseits der Gewebszerstörung, die von T Zellen verursacht wird, entgegen (Niesner et al. 2008), und unterstützt anderseits die Persistenz der Zellen im Gewebe durch die Induktion der microRNA148a, die die Expression des pro-apoptotischen Proteins Bim reguliert (Haftmann et al. 2015). Die Beteiligung von Twist 1 an der Regulation metabolischer Prozesse konnte

bereits nachgewiesen werden. In Myozyten beeinflusst Twist 1 die Speicherung von Glycogen (Mudry et al. 2015a). In Adipozyten induziert Twist 1 die Expression von *Carnitine palmitoyltransferase I* (CPT1) mRNA, einem Schlüsselenzym der Fettsäureoxidation. Außerdem stimuliert Twist 1 die Fettsäureoxidation (Pettersson et al. 2010; Dobrian 2012).

In dieser Arbeit zeigen wir durch die konditionelle genetische Inaktivierung von Twist 1 in CD4+ T Lymphozyten, dessen Einfluss auf die Regulation des Metabolismus von CD4+ T Lymphozyten bei chronischen Entzündungen. Dabei wird die Glycolyse verringert und vermehrt Fettsäuren synthetisiert, um die Zellen vor reaktiven oxidierenden Spezies (ROS) zu schützen. Twist 1 reguliert die Expression von *Myc* herab und fördert die vermehrte Expression von Genen, die in die mitochondriale Biogenese und Fettsäuresynthese involviert sind: *Atf5, Bnip3, Gpt2, Gpx4, Myc, Oxct1, Rdh11, Slc2a1, Slc2a3, Paics, Pfkp,* and *Ppp1r3b*. Zusätzlich konnten wir nachweisen, dass mehrfach re-stimulierte, Twist-defiziente Th1 Zellen unfähig sind, durch Fettsäureoxydation zu überleben, sondern den Stoffwechsel auf Lipid-Peroxidierung umstellen.

1 Introduction

1.1 Adaptive immunity

Recombinatorial rearrangement of different immunoglobulin or T cell receptor gene (TCR) segments is common to all jawed vertebrates. It gives rise to an antigen receptor repertoire that is unique for each and every lymphocyte. After recognition of their specific peptide, lymphocytes respond by clonal amplification, cellular differentiation and production of antibodies to combat pathogens. Importantly, after antigen clearance, a subset of lymphocytes survives to become memory cells. Memory cells are capable of a highly specific recall reaction allowing fast clearance upon re-encounter of the antigen. The lymphocyte population consists of two major subsets, B- and T- cells, that develop from bone-marrow derived hematopoietic progenitor cells, which mature in a bone marrow (Cooper 2015) and a thymus (Shah and Zuniga-Pflucker 2014), respectively. Lymphocytes are distinguished by structurally variable antigen receptors: B-cell antigen receptor (BCR) is formed by the same genes that encode antibodies, class of proteins called immunoglobulins(Ig). The focus of this thesis is cells expressing T cell antigen receptor (TCR), described further.

1.1.1 T cell receptor formation

The T cell receptor (TCR) is generated by random non-homologous DNA recombination of V, D and J gene segments during T- cell development in the thymus. Combinatorial and junctional diversity together with a combination of variable chains gives rise to approximately 10^{18} unique TCRs (Davis and Bjorkman 1988) that become DNA imprinted, allowing clonal expansion of identical cell clones. The maturation of T cells in the thymus leads to the expression of CD3. During the following positive selection T cells that recognize self MHC class I (expressed by all nucleated cells) or II (expressed exclusively on antigen presenting cells) with low avidity are preserved and subsequently express cluster of differentiation (CD) 8+ or CD4+, named cytotoxic T cells (Tc) also called T helper cells (Th), respectively. In the course of the negative selection that follows, cells that strongly bind self-peptide in the context of self-MHC are deleted via induction of apoptosis (Carpenter and Bosselut 2010) or become anergic within the thymus to prevent autoimmunity (Abbas et al. 2007; Germain

2002; Spits 2002). The resulting mature T cell repertoire is tolerant to self-antigens, nevertheless some of the T cells that recognize self-antigen with intermediate avidity are not deleted but become natural T regulatory (nTreg) cells, which have the ability to suppress effector T cell function (Hsieh et al. 2012; Liston et al. 2008). In some cases auto-reactive cells escape these mechanisms and can become, in combination with environmental factors, activated in the periphery leading to development of autoimmune diseases, which is the subject of this thesis and discussed in more detail in section 1.3.

1.1.2 Activation of T cell via T-cell receptor

Mature but naïve T cells leave the thymus and migrate through the SLO (secondary lymphoid organs such as lymph nodes, spleen, tonsils or mucosa-associated lymphatic tissue) and periphery until they encounter TCR respondent antigen displayed by professional antigen-presenting cells (APCs). APCs are comprised of the innate immune system cells, such as dendritic cells (DC) and macrophages but also cells of the adaptive immune system, such as B cells. After a pathogen is captured by an APC and processed in endo-lysosomal compartments (Abbas et al. 2007; Gao et al. 2002; Lustgarten et al. 1991), the APCs migrate along the lymphatics into the T cell zone of the local SLO where they express peptide-MHC molecules binding to TCR together with co-stimulatory molecules such as B7-1 (CD80) and B7-2 (CD86) that are binding to CD28 receptors on T cells. Signalling complex is further stabilized by expression of adhesion molecules, such as leukocyte function-associated antigen-1 (LFA-1) binding to intercellular adhesion molecule-1 (ICAM-1), expressed on the surface of APCs (Huppa and Davis 2003). Timing and duration of signals from surface inhibitory molecules, like cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and programmed cell death protein 1 (PD-1), or stimulatory signals, such as inducible T cell co-stimulator ICOS, influence signalling outcome. TCR triggering without co-stimulation or provision of cytokine signal without TCR stimulus results in T cell anergy (Jenkins and Schwartz 1987; Sckisel et al. 2015). After the successful activation, an intracellular signalling is propagated via the cytosolic region of the TCR. Triggering intracellular activation cascade (Abbas et al. 2007) leads to a burst-like release of Ca^{2++} into cytosol from its stores in the Endoplasmic Reticulum (ER). Calcium release is followed up by expression of canonical transcription factors such as nuclear factor-κB (NF-κB), activator protein 1 (AP-1) and nuclear factor of activated T cells (NFAT). Those factors play in concert with effects of co-stimulatory signalling initiated by CD28 engagement leading to upregulation of the phosphoinositide 3- kinase (PI3K) pathway leading to an activation phenotype (Okkenhaug et al. 2004). Activated cells decrease expression of surface molecules, such as CCR7, and

CD62L, which are SLO localization signals, and up-regulate molecules, such as LFA-1 or CD99 that allow them to emigrate to the site of inflammation via transendothelial migration (Muller 2013). Activated cells express Interleukin 2 receptor-α chain (CD25), which allows them to react to the growth promoting cytokine IL2 (Iezzi et al.). Clonally expanding T cells activate B cells, macrophages and dendritic cells triggering activation of adjacent inflammatory cells.

1.1.3 Differentiation of effector T cells

Post-activation T cell function is defined primarily by expression of a cluster of differentiation (CD) surface molecules. CD8+ T cells specifically eliminate pathogen-infected host cells by cytotoxic means. Meanwhile, CD4+ T cells provide co-stimulatory signals and generate cytokines and chemokines. Cytokines and chemokines provide proliferative and activation signals to neighbour cells, such as CD8+ T cells, B cells (production of antigen-specific antibodies) or macrophages to perform specific functions or recruit new immune cell subsets to the site of inflection. Cytokine milieu, type and duration of TCR engagement, type of APC, nutrient availability and surrounding cells are main factors dictating cell differentiation into a distinct T helper subsets (van Panhuys et al. 2014; van der Windt and Pearce 2012). T helper subsets are defined by expression of a master transcription factor, set of cytokines and functions. Apart from the main recognized subsets (

Figure **1-1**) it is becoming clear that the factors determining phenotype are complex and that there exist number of phenotypically and functionally flexible T helper subsets (Hegazy et al. 2010; Abromson-Leeman et al. 2009; Sakuraba et al. 2009) that are not falling to any established category (Becattini et al. 2015; Hirahara and Nakayama 2016).

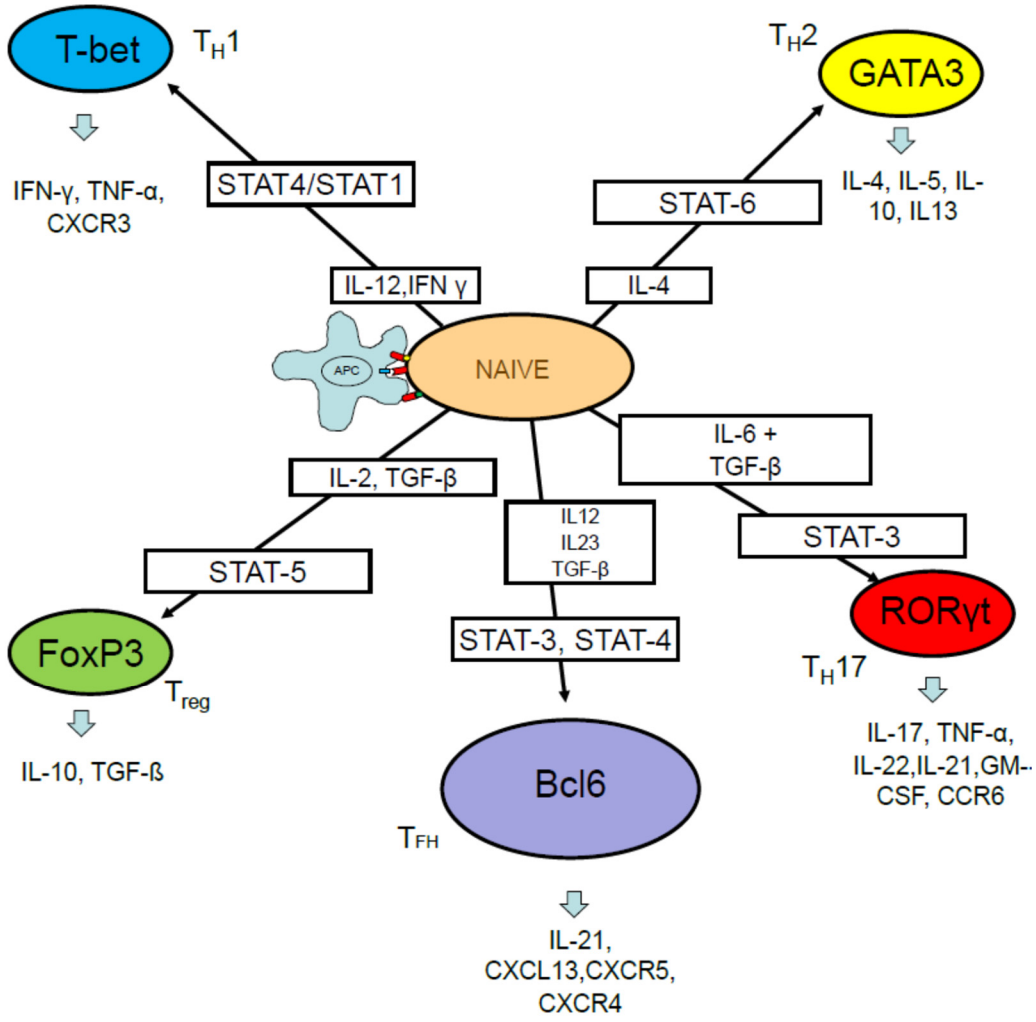

Figure 1-1: T helper subsets:

Depending on the cytokine environment produced by APCs, T helper subsets express exclusive master transcription factors, produce distinguishable cytokines and are involved in distinct immune functions

1.1.4 Effector T helper 1 cells (Th1)

Differentiation into the Th1 lineage is characterized by expression of the master transcription factor T-bet. T-bet expression is induced in two waves: first by TCR engagement in the presence of IFN-γ that activates STAT1, followed with a second wave of antigen-independent regulation by IL-12, signal that induces STAT4 (Szabo et al. 2000; Schulz et al. 2009; Mullen et al. 2001; Afkarian et al. 2002; Lighvani et al. 2001). T-bet upregulates the transcription of *IL-12Rß2* and *IFN-γ* and therefore drives an auto-regulatory positive feedback-loop (Chang and Aune 2005; Schulz et al. 2009). Additionally, expression of T-bet, directly and indirectly, represses genes of other T helper subsets to stabilize the Th1 fate

(Lazarevic and Glimcher 2011; Szabo et al. 2000; Hwang et al. 2005; Djuretic et al. 2007; Naoe et al. 2007). IFN-γ is considered to be a signature cytokine for the Th1 subset (Mosmann et al. 1986; Szabo et al. 2002) and together with the secretion of IL-2 and TNF-α it facilitates immune responses of cytotoxic T cells and macrophages against intracellular viral or bacterial pathogens as well as tumour cells. Additionally, Th1 cells support B cells to secret high-affinity pathogen-specific antibodies. Th1 cells are able to produce IL10, a cytokine with a broad spectrum of anti-inflammatory functions, which are essential as a negative feedback mechanism in the self-limitation of inflammatory responses (Saraiva et al. 2009). IL10 regulates T cell responses by inhibition of MHC class II and co-stimulatory molecule B7-1/B7-2 expression on monocytes and macrophages. Also, IL-10 limits the production of pro-inflammatory cytokines such as IL-1, IL-6, IL-12, IL-18, TNF and various chemokines (Couper et al. 2008). IL-10 can also act directly on CD4 T cells, inhibiting proliferation and production of IL-2, IFN-γ, IL-4, IL-5 and TNF. Several factors, including IL-27 (Anderson et al. 2009), IL-12 (Chang et al. 2007), TGF-β (Xu et al. 2009) or the Notch pathway (Rutz et al. 2008) induce IL10 expression from effector T cells. The transcriptional regulation of IL10 is still not fully understood, but Blimp1 has been shown to induce IL10 expression in Th1 cells in a STAT4 dependent manner (Neumann et al. 2014), while AhR directly acts on IL-10 expression in Th1 phenotypically related Tr1 cells (Mascanfroni et al. 2015).

1.1.5 Memory Th1 cells

The elimination of the antigen and the ensuing reduction of survival factors, as well as intrinsic inhibitory pathways, limit the magnitude and duration of the immune response. In the end, T cell responses decline and the population contracts through apoptosis. A small fraction of cells that survive the activation-induced stress (such as exposure to ROS) or metabolic exhaustion become memory T cells. Upon re-exposure to the same pathogen, memory cells can swiftly clonally expand and clear the antigen. The exact mechanisms of Th1 memory subset formation are still under investigation. At the peak of the primary Th1 response the cell population is heterogeneous. Fate decision to become memory or short-lived effector cell lies in the early expression of the IL2 receptor CD25 (Pepper et al. 2011). When CD25 is expressed, IL2 binding activates STAT5 that induces expression of IL2RB, T-bet, and Blimp1. Cells express low amounts of Bcl6 and downregulate CXCR5, becoming effector T cells. If CD25 is low, Bcl6 is upregulated, T-bet is transiently downregulated and CXCR5-positive population is formed. This population (about 25%) highly resembles the follicular T helper cells (T_{FH}). If cells have low expression of PD1, they will become memory Th1 cells (though some plasticity is retained). If cells retain high PD1 expression, they

become T_{FH} cells (Pepper et al. 2011). In a different study, a persistently stimulated population of memory CD4 Th1 cells in *Mycobacterium tuberculosis* - infected animals was heterogeneous and expressed either KLRG1 or PD-1. Even though KLRG1+ cells express high levels of cytokines in comparison to PD1+ cells, they are short-lived and proliferate poorly. Instead, PD1+ cells express fewer cytokines, retain plasticity and are able to survive longer. Even if this population of PD1+ cells shares many features with follicular helper cells, unlike follicular helper cells, these cells are capable of mounting enormous cytokine response (Reiley et al. 2010).

1.2 Metabolism of T cell

1.2.1 Metabolism during T cell activation

Main metabolic pathways in the cell are depicted in Figure 1-2. During thymus maturation, cells undergo several metabolic switches. Mature naive T cells are maintained by IL-7 that supports homeostatic proliferation and survival (Schluns et al. 2000). IL-7 also supports employment of the oxidative phosphorylation, that is fuelled by catabolic metabolism of fatty acids, glutamine, and glucose (Pearce and Pearce 2013). Upon activation, cytosolic Ca^{2+} released from the ER induces mitochondrial enzymes such as pyruvate dehydrogenase (PDH), an enzyme that catalyse the entrance of glucose-derived pyruvate into the TCA cycle in mitochondria. This initial metabolic phase is necessary for initiation of rapid cell expansion (Sena et al. 2013), during which demand for organelle biosynthesis and synthesis of effector and signalling molecules increases. Increase in oxidative phosphorylation comes together with high levels of reactive oxygen (ROS), produced by the electron transport chain. Next to oxidative phosphorylation, the second most important source of ROS (i.e., hydrogen peroxide and superoxide anion production) is NADPH dependent oxidase NOX5 (D'Autreaux and Toledano 2007; Winterbourn 2008). ROS are necessary for proper cell activation, signalling and T cell function (MacIver et al. 2013; Pearce et al. 2009; Wang et al. 2011). However, if ROS are not scavenged in a timely manner, they have a detrimental impact on the organism. Short-lived oxygen-containing molecules have high chemical reactivity towards DNA, lipids and lipoproteins (Marnett 2002), potentially leading to apoptosis. Conversion of reduced glutathione to its oxidized form by ROS with H_2O as a byproduct is one of the main mechanisms of ROS scavenging and release of oxidative stress, mediated by glutathione peroxidases. One of its major family members, Gpx4, functions as a repressor of 12/15-

lipoxygenase–induced lipid peroxidation (Seiler et al. 2008). It catalyses the reduction of lipid peroxide at the expense of reduced glutathione (Yang et al. 2014). NADPH has the ability to serve as a principal reductant of oxidized glutathione in the cell. Oxidized glutathione is generated during scavenging reactions by Gpx4 (further discussed in 1.3.4).

Biomolecule precursors to fuel cytokine release and proliferation are supplied largely by upregulation of glucose uptake and glycolysis, processes regulated downstream of activation- induced TCR signalling. TCR and CD28 engagement mediates an up-regulation of Akt and phosphoinositide 3-kinase (PI3K) pathway signalling that in the presence of IL-2 lead to activation of mammalian target of rapamycin (mTOR). mTOR is a conserved serine/threonine kinase that plays a crucial role in the control of cell fate decisions both in the steady and activated state. mTOR serves as a node to coordinate signalling, metabolic and migratory pathways (MacIver et al. 2013). During activation, mTOR promotes lipid and protein synthesis, and importantly promotes upregulation of transcription factor hypoxia-inducible factor (HIF1- α) and Myc. HIF1-α is stabilized by low oxygen levels but stabilization also occurs under normoxic conditions in response to T- cell activation via STAT3 signals that are integrated by mTOR pathway, promoting its stability (Dang et al. 2011). Myc and HIF1-α upregulate genes involved in glycolysis and glucose uptake such as Glut-1, phosphofructokinase 1, hexokinase, pyruvate kinase or lactate dehydrogenases (Doedens et al. 2013; Wang et al. 2011; Finlay et al. 2012; Frauwirth et al. 2002), resulting in an increase in glucose uptake and catabolic metabolism (Maciver et al. 2008). These transcriptional events are leading to the initiation of a fast process called aerobic glycolysis, also named Warburg effect (Warburg et al. 1958). It provides cells with energetically inefficient (2 molecules of ATP per molecule of glucose), but rapid and macromolecule rich metabolic resource that supplies cellular replication and new organelles. As a result, cells produce large amounts of lactate, responsible for acidification of the cellular outer space. Failure of T cells to increase glucose and glutamine uptake and metabolism via the PI3K/ Akt/ mTOR/ Myc/ HIF1-α pathway results in decreased survival, cytokine secretion and cytokine production (Michalek et al. 2011; Yaqoob and Calder 1997; Chang et al. 2013). An important aspect of glycolysis is the role of glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in cytokine regulation during T cell activation. It was shown in Th1 cells that GAPDH binds to mRNA encoding IFN-γ, repressing its translation. As soon as glycolysis is triggered, inhibitory GAPDH is dissociated from the IFN-γ promoter and IFN-γ is highly expressed (Chang et al. 2013). Activation of TCR boosts also uptake of alternative carbon sources, most notably glutamine (Levring et al. 2012). Glutamine derived α-ketoglutarate is incorporated into the TCA cycle by an enzyme α-ketoglutarate dehydrogenase (KGDH), which is upregulated upon activation by increased calcium influx.

TCA generates principle electron donors $FADH_2$, NADH, and NAD+ in the TCA cycle, supplying to the electron transport chain that is fuelling OXPHOS. Glutamine contribution to TCA also supports the biosynthesis of polyamines and fatty acids. Fatty acids are formed from TCA cycle intermediates and NADPH molecules derived from the pentose phosphate pathway in anaplerosis process called fatty acid synthesis (FAS). Fatty acids can be further processed to form cholesterol that is used for membrane build-up during proliferation, phospholipids used as cell signalling molecules or epigenetic modification marks. Fatty acids can be stored in a form of triglycerides (Chou et al. 2013) and be oxidized to form energy during periods of quiescence (Pearce et al. 2013). It is established, that synthesis and maintenance of cellular lipid levels permit rapid membrane biogenesis and cell cycle progression, survival and effector function (Kidani et al. 2013), but the molecular pathways underlying these processes remain poorly defined. FAS has been shown to be regulated coordinately by activation-induced bHLH transcription factors mTOR, c-Myc (Wang et al. 2011) and PPAR (Grygiel-Gorniak 2014) in T cells. Following activation, Myc and mTOR coordinate SREBP bHLH transcription factors (Kidani et al. 2013) that regulate lipid storage through regulation of glycolytic and lipogenic enzymes, such as hexokinase, fatty acid synthase and most importantly, Acetyl-CoA carboxylase (ACC) (Horton et al. 2002). ACC converts Acetyl-CoA into Malonyl-CoA, a precursor in the synthesis of fatty acids in TCA. Malonyl-CoA provides the two-carbon building blocks that are used to create larger fatty acids by condensation with Acetyl- CoA, NADPH dependent reaction catalyzed by fatty acid synthase (FASN). Glucose alone stimulates lipid genesis by stimulation of hexokinase expression, fatty acid synthase and ACC-2 proteins (Guillet-Deniau et al. 2004). Subsequently, an elevated flux of glucose into the cell leads to an increased de *novo* lipogenesis with triglyceride accumulation (Visiedo et al. 2013).

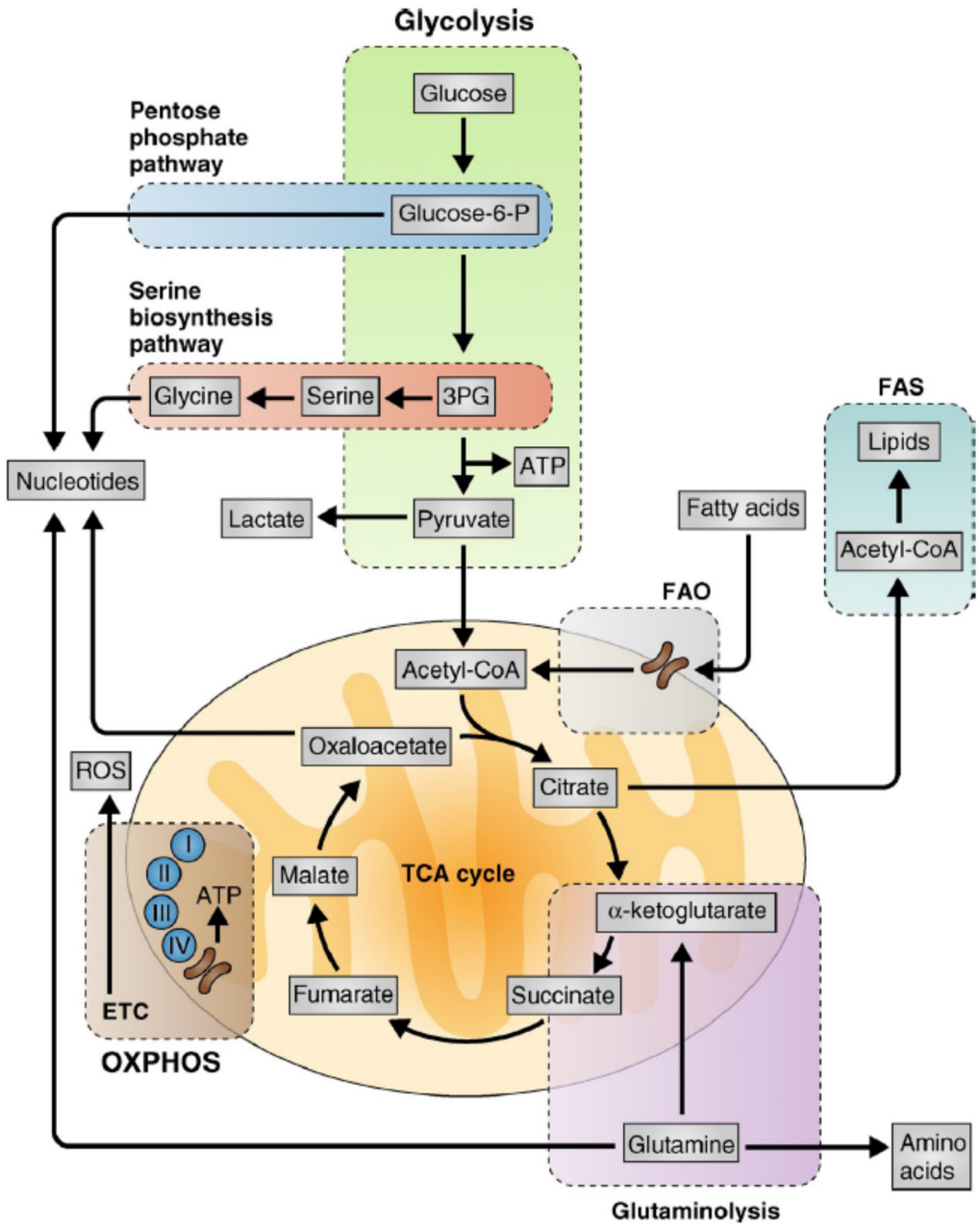

Figure 1-2. Mayor metabolic pathways in the cell.

Adapted from (Buck et al. 2015)

1.2.2 Metabolism of resting T cells

During an activation-induced fatty acid synthesis in effector T cells, production of Malonyl-CoA inhibits fatty acid oxidation (Hopkins et al. 2003). When an antigen is cleared, most effector T cells die, leaving behind only a small population of memory T cells that need to

survive long periods of quiescence. During this phase, the splicing variant of Bnip3 has been shown to promote mitophagy that supports the formation of memory (O'Sullivan et al. 2015; Gang et al. 2015) while protecting against oxidative stress (Chinnadurai et al. 2008). Memory T cells in many ways resemble naive T cells but differences have been shown. In terms of biological function, it has been suggested that levels of spare respiratory capacity (SRC) and mitochondrial mass are higher in CD8 memory T cells (van der Windt et al. 2012), providing the extra energy storage to promote long-term survival and flexibility to use diverse substrates for energy generation, such as the oxidation of fatty acids. Bigger the mitochondria with higher SRC, bigger the ability of memory cells to simultaneously synthesize and oxidize fatty acids and therefore maintain mitochondrial fitness for a oxidative production of ATP in times of stress and metabolic deprivation. High SRC is a phenotypic trait connected to the longevity and the ability to robustly expand upon antigen stimulation (Pearce et al. 2009; van der Windt et al. 2012; van der Windt et al. 2013). Understanding the regulation of FAO is still in the beginning stages. AMPK signaling has been shown to be crucial in a metabolic regulation of CD8 memory generation (Rolf et al. 2013), suppressing mTOR signaling (MacIver et al. 2013). AMPK signaling serves as an energy stress sensor that is increased by a change in intracellular AMP to ATP concentration. AMPK induces CPT1 expression (van der Windt and Pearce 2012), possibly in a peroxisome proliferator activated receptor (PPAR) gamma coactivator (PGC)-1 dependent mechanism, as demonstrated in muscle cells (Jager et al. 2007; Lee et al. 2006). AMPK also phosphorylates and inhibits ACC, a crucial enzyme for production Malonyl-CoA. Low levels of Malonyl-CoA release inhibition of Carnitine palmitoyl transferase 1a (CPT1) facilitating fatty acid oxidation (Jones and Thompson 2007). Furthermore, regulation of fatty acid oxidation by the PD-1 receptor engagement has been shown (Patsoukis et al. 2015). PD1 engagement by PD1-L induces a decrease in aerobic glycolysis and enhances FAO in activated T cells. Such metabolic switch is mediated via increased expression of CPT1A together with upregulation of TAG hydrolase desnutrin/adipocyte triglyceride lipase (ATGL), inducing lipolysis (Patsoukis et al. 2015). Currently, the source of fatty acids for oxidation in T memory cells is debated and probably might be a distinguishing feature in metabolic cell phenotypes. CD8 memory T cells have been found to be unable to uptake fatty acids from their extracellular environment as effector T cells do, and are dependent on intracellularly *de novo* synthesis of fatty acids, where glucose serves as one of the important sources for fatty acid synthesis (Weinberg and Chandel 2014; O'Sullivan et al. 2014). Surprisingly, at the same time, fatty acids are accessed for oxidation, a parallel and opposing process called a “futile cycle” (Weinberg and Chandel 2014). While the futility of T cell memory is just an emerging concept, it has been shown that a driving factor in futility (cycling of substrates between *de novo* lipogenesis and lipid oxidation) is the hormone leptin (Dulloo et al. 2004).

Leptin is produced by adipocytes and has been shown to be increasingly present in inflamed joints of arthritic patients, where its levels are correlated with disease activity. Leptin modulation of AMPK/ acetyl-CoA carboxylase (ACC) and mTOR/ ribosomal S6 Kinase was indicated in the process (Tian et al. 2014).

Formation of triacylglycerol from activation induced glucose uptake is consistent with the critical role of Notch as a mediator of glucose uptake, for memory T cell survival (Maekawa et al. 2015). Further evidence for intracellularly accessed fat storage in memory T cells also comes from observation that LAL, enzyme hydrolyzing cholesterol esters and triacylglycerol to generate free FA (Sheriff et al. 1995) is highly expressed in CD8 memory T cells, while CD36, the main fatty acid uptake molecule is downregulated in memory T cells, in contrast to their effector counterparts (Sheriff et al. 1995) and that intracellular TAG synthesis supports cell longevity (Cui et al. 2015). Of note, most of the T cell metabolic studies described deal with CD8 T cells metabolism, while CD4 metabolism still remains not explored.

1.2.3 T cell differentiation is associated with distinct metabolic pathways

Metabolism influences the T cell phenotype by modulating both lineage-specific differentiation and cytokine production. A simplified view that glycolysis leads to the more inflammatory phenotype of effector cells, while oxidative phosphorylation and FAO is connected with non-inflammatory cells of T-reg and T_{FH} (Pearce and Pearce 2013; Michalek et al. 2011) is challenged by recent research showing high metabolic flexibility among cell types. Conventional T cells are activated and even are capable of limited proliferation when aerobic glycolysis is limited, as they will switch to OXPHOS (Chang et al. 2013; Sena et al. 2013) and T effector cells still exhibit a substantial rate of FAO upon activation (Michalek et al. 2011). Example of such divergence has been shown in metabolic regulation of cell fate of Th17 versus T-reg population. In Th17 cells HIF1-α, the promoter of glycolysis, binds and activates RORγ-t, simultaneously attenuating development of T-reg cells by binding FoxP3 and targeting it for proteasomal degradation (Dang et al. 2011). Accordingly, T-reg cells were considered to be a cell type-dependent solely on fatty acid oxidation, as *in vitro* blockade of CPT1a disrupts T- reg generation, while supplementation with fatty acids *in vitro* supports T- reg differentiation and function (Berod et al. 2014; Michalek et al. 2011). However, recently it was shown that transient expression of glycolysis enzyme Enolase-1 is critical for the induction of potent iT-regs from conventional T cells, even if a long-term expression of Enolase 1 can become detrimental (De Rosa et al. 2015), pointing to a complex metabolic phenotype of not only T-reg cells but probably also of other cell phenotypes. Bcl6, lineage-defining transcription factor of T_{FH}, suppresses several glycolysis

genes potentiated by c-Myc and HIF1-α expression in Th1 cells (Oestreich et al. 2014), which are reliant in survival and effector function on glycolysis (Michalek et al. 2011; Chang et al. 2013). T-box transcription factor T-bet is required for IL2-STAT5 dependent increase of glycolytic enzyme expression (Oestreich et al. 2014) while it also antagonizes Bcl6 mediated gene repression of genes encoding molecules in the glycolysis pathway by direct interaction with its DNA binding domain.

1.3 Rheumatic disease

Juvenile idiopathic arthritis (JIA) is the rheumatoid disease (RD), which is closely related to another chronic autoimmune pathology Rheumatoid arthritis (RA) and other diseases such as psoriasis arthritis (PsA), ankylosing spondylitis (AS) or Sjögren's disease (SD) (Yildirim et al. 2012). JIA is a highly heterogeneous disease encompassing arthritis that lasts at least 6 weeks with onset before the age of 16 years (Ravelli and Martini 2007). JIA has several phenotypically and genetically distinct subtypes (Giancane et al. 2016) and unified diagnosis is still under debate (Martini 2012). Rheumatic joint inflammation presents itself by a formation of a thick multilayered granulation tissue in the joint, termed pannus, which is affecting tendons, cartilage and bone resulting in progressive joint and soft tissue destruction. In RA, shortened life expectancy is associated with increased cardiovascular risk, metabolic syndrome, psychosocial deficit including psychiatric disease, osteoporosis, and increased cancer rates (Malmstrom et al. 2017). The etiology of rheumatoid diseases is highly complex and not fully elucidated. However, genetic factors, leading to a break in peripheral tolerance during thymus T cell maturation, are considered as a prerequisite to its early development (van der Woude et al. 2009). Several genetic alterations have been discovered to be associated with increased risk for arthritic synovial inflammation: well known genetic alterations is to the gene coding for the MHC class II molecule human leukocyte antigen D related (HLA-DR) on chromosome 6. Every clinical subtype of JIA is associated with distinct set of HLA haplotype, but RA has been associated with haplotypes HLA DRB1 and HLA DRB4. Some of the other contributing mutations are: single nucleotide polymorphisms in the genes encoding for protein tyrosine phosphatase non-receptor type 22 (van Oene et al. 2005; Pierer et al. 2006), or single polymorphisms found in the gene encoding programmed death-1 (PD-1). Resulting alterations in the activity of proteins lead to a decreased elimination of autoreactive T cells (Lin et al. 2004; Prokunina et al. 2004). Autoreactive T cells become increasingly present in the periphery while T-reg cell numbers decrease (Filion et al. 1995; Villoslada et al. 2001; Danke et al. 2004). Additionally, polymorphisms of the macrophage-resistance gene NRAMP1 that regulates activation of

macrophages for enhanced expression of tumor necrosis factor (TNF)-α, IL1-β and MHC class II, shared between RA and JIA patients might contribute to the development of the disease (Sanjeevi et al. 2000). Polymorphisms in PADI4 enzyme that are together with smoking responsible for the posttranslational conversion of arginine residues into citrulline have been found in both RA and JIA patients (Hisa et al. 2017). Citrullination is thought to improve the fit in HLA alleles (Huizinga et al. 2005) and results in antibodies against post-translationally citrullinated protein epitopes (ACPAs) (Kokkonen et al. 2011; Klareskog et al. 2006; Kocijan et al. 2013). In some cases, antibodies against the Fc portion of IgG ("rheumatoid factor") can be detected. Disease outbreak often begins with a triggering event, which is often a bacterial or viral infection with generally subclinical symptoms (Lang et al. 2002; Zipris et al. 2005).

1.3.1 Chronically stimulated CD4+ T cells in Rheumatoid Arthritis

Histological staining of an inflamed synovial tissue reveals follicular lymphoid-like structures resembling germinal centres of lymphoid organs. These aggregates are rich in T cells, B cells expressing MHC class II, and dendritic cells (Simon et al. 1994). $CD4^+$ T cells most prominent lymphocytic cell type infiltrating the inflamed synovium (Panayi et al. 1992). Physiologically, memory T cells that recognize pathogenic antigens provide the organism with long-lasting protection against recurrent infections. However, in the case of rheumatoid disease T cell longevity and the persistent effector response to autoantigens becomes detrimental. The important role of CD4+ T helper cells in autoimmune pathology is well demonstrated in depletion studies of T helper cells in murine models of autoimmune arthritis (Hom et al. 1988; Ranges et al. 1985). CD4+ T cells are initially activated by cognate cartilage antigen, citrullinated peptides or TCR independent pro-inflammatory cytokines that are secreted by neighbouring cells (Sattler et al. ; Zhang et al. ; Brennan et al. 2002; Burkhardt et al. 2006; Huizinga et al. 2005). Past or continuous antigenic stimulation is reflected in a CD45RO+ CD45RB - memory phenotype of *ex vivo* isolated CD4+ T cells. Further, the inflammatory and invasive rheumatoid arthritis synovial tissue is characterized by elevated levels of inflammatory T-helper cell 1 (Th1) cytokines such as IL-1β and TNFα (Feldmann et al. 2001), although the presence and inflammatory role of Th17 cells are also debated (Koenders et al. 2006). Transcriptionally, T cells isolated from synovial fluid of rheumatoid arthritis patient's express high levels of the transcription factor TWIST1, a hallmark of repeated stimulation of Th1 cell´s TCR receptor (Niesner et al.).T wist1 also contributes to the survival and persistence of CD4+ T cells that are perpetrating synovial

inflammation (James et al. 2014; Firestein 2003; Haftmann et al. 2015). Other contributing factors to the survival, such as the inflammatory milieu (Cope 2002). CD4+ T helper cells were also found to be hypo-responsive to TCR stimulation in inflamed tissues (Reyes et al.), to which contributes chronic stimulation and high levels of oxidants at the site of inflammation (Maurice et al. ; Bao et al.). Hyporesponsiveness of synovial CD4+ T cells is reflected in downregulation of CD28 (Ammirati et al. ; Baniyash ; Zhang et al. ; Cope 2002) and by expression of negative regulators of co-stimulation such as CTLA-1 or programmed cell death 1 (PD-1) (Li et al. 2014; Wan et al. 2006). PD1 is a key inhibitory cell-surface receptor of the CD28 superfamily that triggers pathways to attenuate T-cell responses and promote T-cell tolerance. Rao et al has shown that an expanded population of CD4+ T cells, isolated from synovial fluid of patients with rheumatoid disease express PD1+, secrete IFN-γ and IL21 and is CXCR5-, therefore are not typical follicular helper cells but are capable pathogenic B cell instruction to secrete auto-antibodies and therefore are considered as pathogenic memory T cells (Rao et al. 2017b).

1.3.2 Metabolism of autoimmune cells

Due to their longevity and persistent memory response, pathogenic autoimmune T cells require a strategy for long-lasting energy supply. The metabolic pathways by which CD4+ T cells gain their energy are expected to display unique survival and energetic signatures. Metabolic adaptation of rheumatic synovial T cells has not been studied so far. In 2016 Weyand and colleagues were able to show that naive CD4+ T cells from RA patients' blood have a defect in the key glycolytic enzyme PFKFB3 and are therefore unable to upregulate glycolytic production of ATP in an activated state (Yang et al. 2016). In consequence, cells are forced to increase the shuffle of acquired glucose to the pentose phosphate pathway, which results in the production of increased levels of NAPDH. NAPDH reduces glutathione which is a mayor ROS scavenger. Lower levels of ROS affect intracellular signaling pathways in cells resulting in insufficient activation of the redox-sensitive kinase ataxia telangiectasia mutated (ATM), which leads to a bypass of the G2/M cell cycle checkpoint leading up to a hyper-proliferation and differentiation towards Th1/Th17 phenotype. The study aiming at the chronically stimulated human memory T cells isolated *ex vivo* from autoimmune disease systemic lupus erythematosus (SLE) patients has shown that cells secure ATP production through oxidative phosphorylation while glycolytic activity is significantly lower than in acutely activated cells or lymphocytes from healthy patients (Wahl et al. 2010).

1.3.3 Synovial hypoxia

Thickening of the synovial lining, large numbers of infiltrating T cells, B cells and macrophages, vascular damage and swelling cause interruption of blood supply leading to lower oxygen tensions that are typical in the inflammatory and invasive rheumatoid arthritis (RA). Oxygen tensions range between approximately 2 % - 4.4 % oxygen (18 to 33 mmHg) in inflamed synovium compared with 8.5% to 13.5% (65 to 103 mmHg) in healthy individuals blood (Lewis et al. 1999; Sivakumar et al. 2008). A major transcriptional regulator of low oxygen tension is HIF1-α. HIF1-α belongs to the bHLH protein family and functionally needs to form a heterodimer with a β-subunit. The α-subunit undergoes oxygen-dependent hydroxylation by PHD, resulting in ubiquitination and degradation by the proteasome. Besides O^{2-} dependent hypoxic stabilization of the HIF1-α subunit, other stabilizers of HIF1-α exist in normoxic cells such as TNF-α. TNF-α has been shown to increase the accumulation and transcriptional activity of HIF1-α in a NF-κB dependent manner, without affecting its mRNA (Jung et al. 2003).

1.3.4 Reactive oxygen species in synovial CD4+ T cells

The cell possesses a natural ability to scavenge O^{2-}, H_2O_2 molecules through three main antioxidant enzymes - superoxide dismutase (SOD), catalases (CAT) and glutathione peroxidases (GPX). Nevertheless, if ROS are overproduced and not scavenged, widespread lipid, protein, mitochondrial and DNA damage occurs (Filippin et al. 2008). Moreover, ROS have a high affinity to lipids. Lipid peroxidation was found to be increased in a synoviocytes of RA patients, while the antioxidant status of the cells accessed by levels of GSH and SOD was impaired (Seven et al. 2008). Lipid peroxidation is a major cause of an alternative form of iron-dependent, regulated non-apoptotic cell death, Ferroptosis and is mainly inhibited by the repair enzyme glutathione peroxidase 4 (GPX4) (Yang and Stockwell 2016). GPX4 has been shown to be crucial for Th1 mediated anti-parasitic responses in both normoxia, and hypoxia (Matsushita et al. 2015). So far, levels of GPX4 were not assessed in patients with RA. However glutathione serum levels were reported to be decreased by 50% in patients with RA, compared to healthy serum donors (Hassan et al. 2001).

1.3.5 Proteomic and metabolome analysis of synovial fluid

Synovial fluid (SF) provides both lubrication and nutrition to the joint. Nevertheless, the majority of SF from rheumatologic patients contains IFN-γ (median level 17 pg/ml) in addition

to monokines such as IL-6 (4700 pg/ml) and TNF-α (157 pg/ml) (Steiner et al. 1999). Moreover, rheumatoid synovium is associated with an increased production of adipokines, such as leptin and adiponectin that were identified as relevant factors involved in interactions between metabolism and rheumatic disorders (Conde et al. 2011). In a mass spectrometry proteomic comparative study of proteins upregulated in synovial fluid of a RA joint in comparison to protein expressed in synovial fluid of osteoarthritic patients (Balakrishnan et al. 2014), it was found that an oxidoreductase myeloperoxidase that is associated with oxidative stress in the inflamed joints (Stamp et al. 2012; Balakrishnan et al. 2014) was 16x upregulated in RA synovium in contrast to osteoarthritis. Coro1a, a phagosome protein with a role of in a T cell survival (Mueller et al. 2008) is 6 times higher. Additionally several enzymes involved in the glycolytic pathway were upregulated in synovial fluid of rheumatic patients such as glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase (Henderson et al. 1979), hexokinase (HK3) (Logvinenko Iu et al. 1982; Zborovskaia 1983) glucose phosphate isomerase (GPI) (Schaller et al. 2006), enolase 1 (ENO1) and ALDOA (Balakrishnan et al. 2014). Nevertheless, the cellular source of these enzymes has not been identified and it is probable that glycolysis is highly upregulated in cells that produce large amounts of antibodies, such as plasma cells (Neuman 2015). An abundance of succinate, aspartate, glutamate, and citrulline in synovial fluid from patients with RA attests to involvement of the TCA cycle (Kim et al. 2014). A 2016 study highlighted increased levels of fatty acids and cholesterol and decreased levels of amino acids and glucose in patients with RA (Zhou et al. 2016).

1.4 Twist 1 – the hallmark of repeatedly activated Th1 cells

In various animal models, Th1 cells with a history of repeated activation by (auto-) antigen accumulate in inflamed tissues (Fuss et al. 1996; Morita et al. 1998; Yamada et al. 2008). Similarly, effector memory Th1 cells persisting in the microenvironment of the inflamed tissue (Niesner et al. 2008) were found in patients suffering from chronic inflammatory joint or gut diseases. These cells express high levels of Twist1, a bHLH transcription factor hallmarking repeated stimulation *in vitro* and *in vivo* (Niesner et al. 2008). *In vitro,* Twist1 is gradually upregulated in murine Th1 cells with every round of re-stimulation and its transcription depends on IL-12/STAT4 signaling and NF-κB and NFAT mediated activation. It is considered as a Th1 specific transcription factor. Indeed, relatively to Th1, it is minimally expressed in Th2 or Th17 after repeated activation (Niesner et al. 2008). Twist1 represents an endogenous regulator of pro-inflammatory Th1 cells and is recognized as a suitable biomarker for the pathogenic memory Th1 compartment generated during chronic inflammation (Niesner et al. 2008). Knockdown of Twist1 in Th1 cells used in an adoptive

transfer model of antigen-induced arthritis results in severe inflammation and tissue destruction by cellular infiltrate, the two major characteristics of chronic inflammation.

1.4.1 Molecular properties and function of Twist1

Twist1 is a transcriptional repressor and a member of the basic helix-loop-helix (bHLH) family (Barnes and Firulli 2009). The transcriptional regulatory outcomes of Twist1 are dictated by several factors that include its spatial and temporal cellular expression, protein–protein interactions with other bHLH proteins (Castanon et al. 2001), post-transcriptional regulation by phosphorylation and accessibility of the other E-box proteins (Firulli and Conway 2008). Twist1 has the generic basic- helix- loop- helix (bHLH) modular structure. At the C-terminal region is located the Twist box (WR) (Kadesch 1993) important for its dimerization. GSK3 mediated phosphorylation of WR domain is crucial for dimerization with non-bHLH transcription factors (Lander et al. 2013). The N-terminus region interacts with the E-box sequence (CANNTG) on target genes and is responsible for DNA binding (Massari and Murre 2000). The momentary balance among Twist1, Id, and E proteins is likely important in defining Twist1 activity (Hayashi et al. 2007) throughout binding to is co-factors (Connerney et al. 2006). The level of Twist1 phosphorylation further dictates the transcriptional outcome. PKA, PKC, PKB/Akt, and PP2 can all determine phosphorylation levels of Twist1 at highly conserved Ser and Thr residues in the HLH region (Firulli and Conway 2008). Complexity of potential Twist1 mediated mechanisms contributes to difficult prediction of transcriptional outcome. Twist1 was first shown to play a positive role in dorsoventral patterning in Drosophila embryos (Barnes and Firulli 2009), cancer survival and metastasis (Yang et al. 2008; Kawagoe et al. 2007). In neuroblastoma, Twist1 acts as negative feedback inhibitor of Myc induced apoptosis (Kawagoe et al. 2007). In immune cells, Twist proteins are key regulators of NF-κB– mediated inflammation, as Twist2 deficiency, or haploinsufficiency of Twist1 and Twist2, results in a lethal systemic inflammatory metabolic syndrome linked to enhanced proinflammatory cytokine production (Sosic et al. 2003; Sharif et al. 2006). In Th1 cells Twist1 limits inflammation by suppressing IL2, IFN-γ and TNF-a production (Niesner et al. 2008) by interaction with RunX3, binding partner of T-bet for the activation of the IFN-γ promoter (Pham et al. 2012). Role of Twist1 in metabolism has been described in adipose tissue and muscle cells (Pan et al. 2009; Mudry et al. 2015b; Pettersson et al. 2010). In white adipose tissue, characterized by low grade, long lasting inflammation, Twist1 silencing reduces FAO together with modulation of IL6 expression via reduction of CPT1 (Pettersson et al. 2010). In brown adipocytes Twist1 is a negative feedback regulator of PPAR-δ/PGC1α inhibiting energy expenditure in mitochondria (Pan et al. 2009). Interestingly, Twist1 has

been also shown to inhibit c-Myc-induced ROS accumulation and apoptosis in carcinogenesis (Floc'h et al. 2013).

2 Results

2.1 Chronically activated T cells decrease glucose uptake and glycolysis

In order to study metabolic adaptation to a repeated Th1 cell activation, we employed the established *in vitro* model of Th1 mediated chronic inflammation, described in Methods section 3.2.2 and in Figure 2-1.

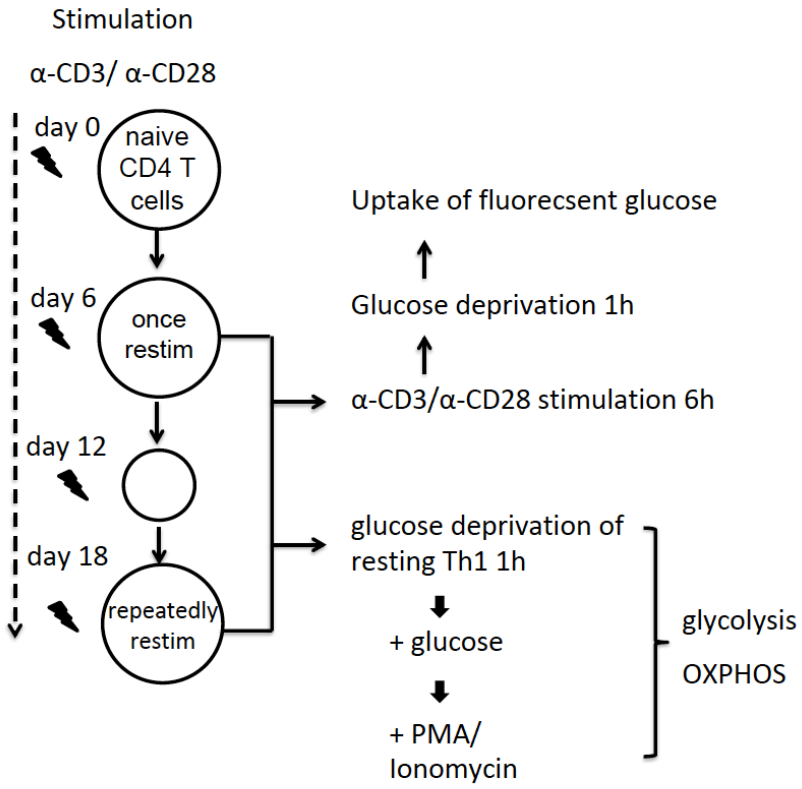

Figure 2-1: *In vitro* chronic inflammation model to study metabolic adaptation:

Schematic representation and experimental set up of an *in vitro* model mimicking effector (once restimulated) and repeatedly restimulated (three times stimulated) differentiation of CD4+ Th1 cells. Right part depicts experimental set-up of metabolic profiling. Glucose uptake was monitored by flow cytometry analysis of fluorescent analogue of glucose (2-NBDG) uptake; glycolysis and oxidative phosphorylation (OXPHOS) was assessed by monitoring of its surrogate markers- acidification rate (ECAR) and oxidative consumption rate (OCR), respectively.

For a long-term Th1 culture, CD4+ CD62L+ cells, defined as naïve, were isolated from CD4-Cre-Twist1$^{wt/wt}$ and CD4-Cre-Twist1$^{loxP/loxP}$ mice. Cells were re-stimulated in 6-day intervals: once - to achieve effector Th1 cell phenotype, or three times - to achieve chronically stimulated, or "pathogenic memory" Th1 cell phenotype. Three times restimulated Th1 lymphocytes might reflect cells that can be found in patients with a chronic autoimmune disease. To study metabolic properties of above-mentioned Th1 phenotypes, viable cells were isolated after 6th day or 18th day of the culture using Ficoll density gradient and their metabolic properties were analyzed. First, to monitor glucose uptake during activation, both phenotypes were re-stimulated for 6 hours with CD3/CD28. Prior to the addition of the fluorescent glucose analogue 2-NBDG, cells were starved of glucose for another 60 min in the glucose-free media. After the addition of fluorescent analogue 2-NBDG, glucose uptake was analyzed by a flow cytometer in FL1 channel. The uptake of glucose was reduced by 2.3-fold in three-times activated Th1 cells compared to Th1 cells which were reactivated once (Figure 2-2, black bars). Controls were done by blocking glucose uptake with Cytochalasin B (gray bars), that blocks entrance of glucose to the cell. They showed that once activated Th1 cells took up 3.5-fold more 2-NBDG than control cells in contrast to repeatedly stimulated Th1 cells that took up in average only 2-fold more 2-NBDG than control cells (Figure 2-2).

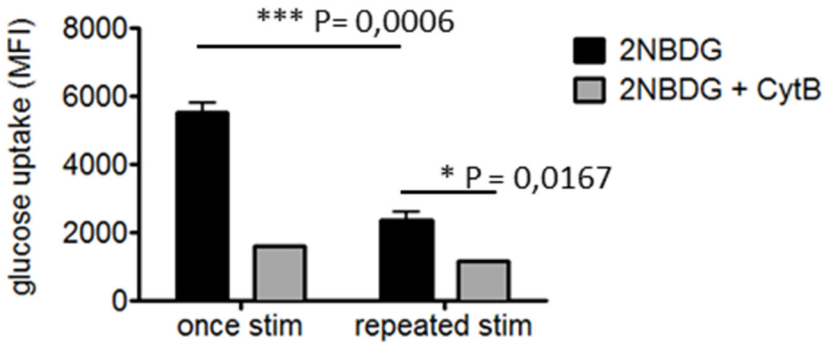

Figure 2-2: Repeatedly stimulated Th1 cells decrease their glucose uptake. :

Glucose uptake is depicted as the mean fluorescence intensity (MFI) of intracellular 2-NBDG molecules of *in vitro* cultured 6 (once re-stimulated) or 18 (three times re-stimulated) days old T_H1 polarized cells. Prior to uptake, cells were restimulated 6h with 3µg/ml of CD3 and 1,5 µg/ml of CD28. Fluorescence was analyzed by FACS after 6h of CD3/CD28 stimulation and subsequent exclusion of PI+ dead cells. Cells treated with Cytochalasin B, glucose transport blocker, were used as an internal background control. Data is presented as mean, with SEM, representative of three independent experiments. Statistics: Mann Whitney test, two tailed

Next, in order to analyse the activity of two major cellular energetic pathways - glycolysis and oxidative phosphorylation, we used Seahorse XF96 analyzer that measures a rate of lactate output and oxidative consumption, its respective indicators. Prior to the measurement, cells were washed several times in glucose-free media and seeded in a glucose-free medium for minimum 1 hour. First, we compared metabolic parameters of cells that have no access to glucose, as resting, non- stimulated cells. In comparison to resting/glucose deprived once stimulated Th1 cells, resting/glucose deprived repeatedly stimulated Th1 cells had minor, nevertheless significant increase in lactate production (Figure 2-3a) and highly increased (2-fold) oxidative consumption rate (Figure 2-3b). After the addition of glucose (Figure 2-3c), we monitored percentage of baseline (baseline= glucose-starved cells ECAR) acidification rates. Upon addition of glucose, once activated Th1 cells upregulated their glycolysis 1.4-fold more compared to repeatedly activated Th1 cells (Figure 2-3c), 0 min. The increased lactate production of once activated Th1 cells was maintained following re-activation with PMA/ Ionomycin (marked with an arrow, 28 min). Although OCR dropped following provision of glucose and re-stimulation with PMA/Ionomycin (arrow PMA/Iono), repeatedly activated Th1 cells maintained higher OCR compared to once activated Th1 cells (Figure 2-3d).

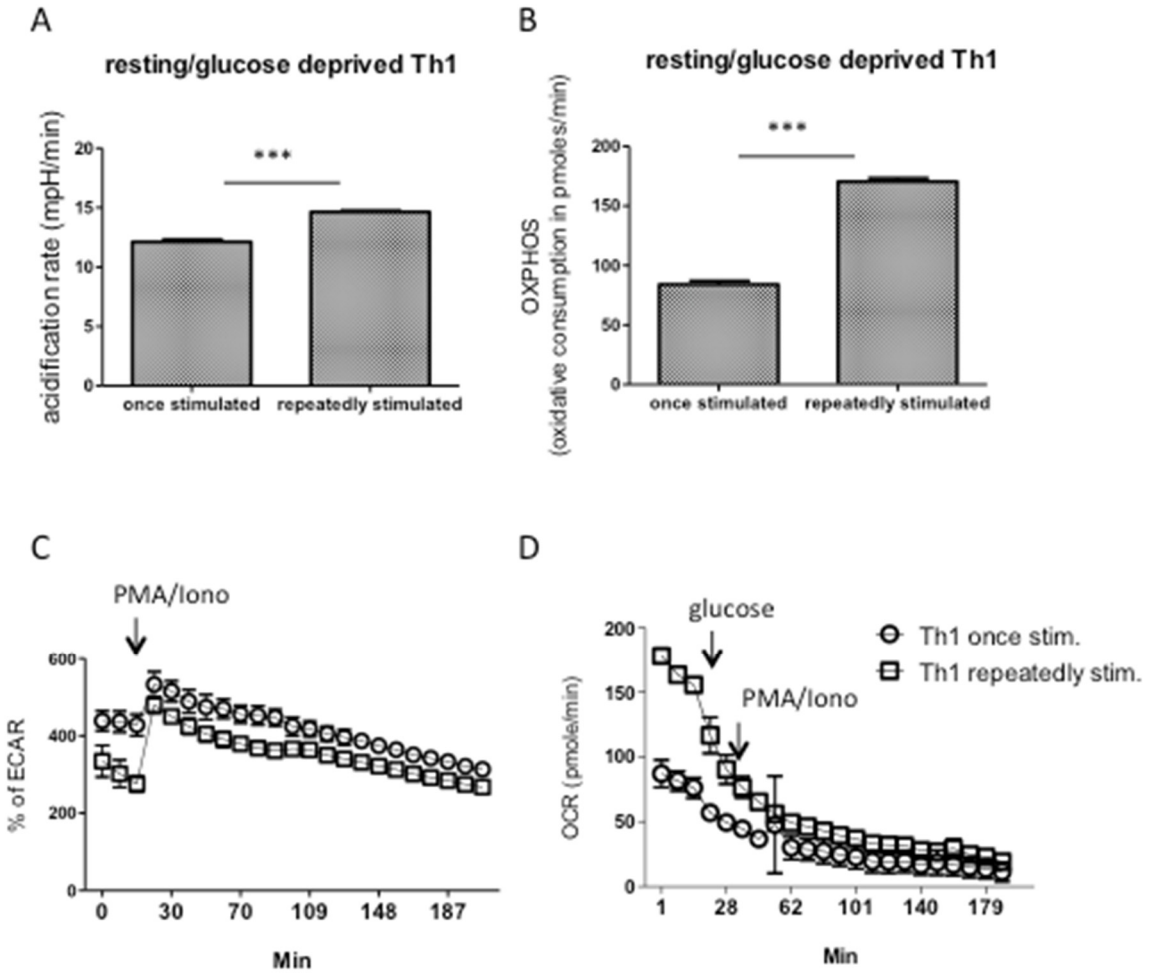

Figure 2-3 After glucose addition, three times stimulated Th1 cells decrease glycolysis and increase oxidative phosphorylation in contrast to once stimulated Th1 cells:

(a) mean value of acidification rate and (b) oxidative consumption rate of *in vitro* cultured, resting, glucose deprived Th1 cells cultured for 6 (once re-stimulated) or 18 (three times re-stimulated) days (c) mean % of extracellular acidification rate (ECAR) derived from supplied glucose before and after stimulation with PMA/Ionomycin (indicated with an arrow) (d) oxygen consumption rate in cells before addition of glucose, after addition of glucose (arrow - glucose) and after stimulation with PMA and Ionomycin (arrow- PMA/Iono). Paired T test $P < 0.0001$ as of 60min after PMA/IONO activation in c,d. Graphs are representative of three independent experiments. Error bars indicate SEM.

Effector T cells were previously shown to depend on glycolysis for activation-induced proliferation, survival and effector function (Michalek et al. 2011; Yaqoob and Calder 1997; Chang et al. 2013). To investigate the level of dependency on glycolysis in once and three-time (repeatedly) stimulated Th1 cells we compared their post-activation survival in the presence of glycolysis inhibitor 2-Deoxy-D-glucose (2DG) (Pelicano et al. 2006). 2- DG is a glucose analogue that is phosphorylated on the 6th position to form 2-DG6P and cannot be

metabolized further by glycolytic enzymes and therefore inhibits glycolysis (Pelicano et al. 2006). A culture of cells with selective glycolysis inhibitor 2DG resulted in 85% decrease in recovery of cell numbers 72 hours after re-activation of once activated Th1 cells compared to a 50% reduction in recovery of repeatedly stimulated Th1 cells (Figure 2-4a). We hypothesized that reduction in glycolysis indicates a shift towards oxidation of fatty acids, as memory CD8 T cells were shown to be dependent on oxidation of fatty acids (Pearce et al. 2009) synthesized intracellularly (Weinberg and Chandel 2014). Nevertheless, dependence on FAO was never shown in memory T cells, or the repeatedly activated CD4+ T cells. To this end, we cultured repeatedly stimulated Th1 cells in a presence of Etomoxir, an inhibitor of carnitine palmitoyltransferase-1 (CPT1) (Horn et al. 2004), the rate-limiting enzyme of fatty acid oxidation. Repeatedly activated Th1 cells did not survive in culture conditions where fatty acid oxidation was inhibited by Etomoxir (Figure 2-4b).

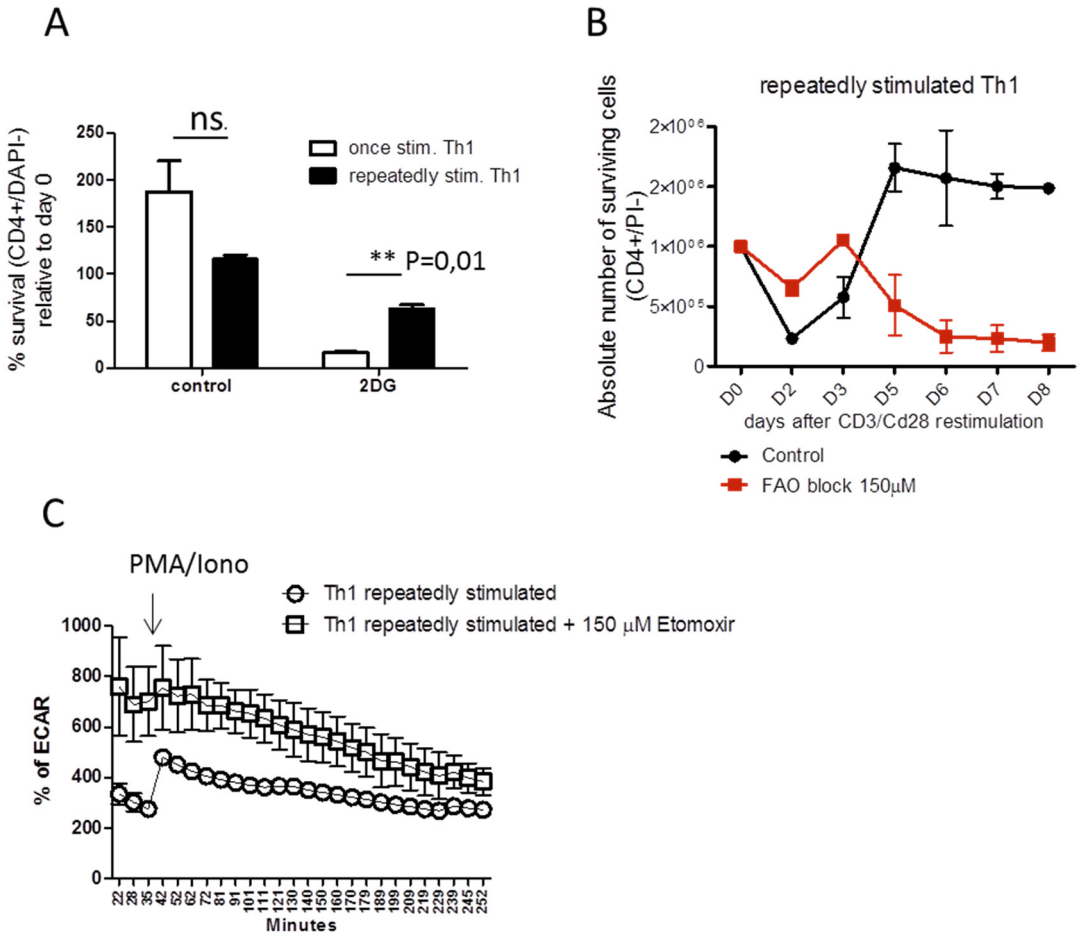

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Figure 2-4: Repeatedly stimulated Th1 cells are less dependent on glucose for post-activation survival and need oxidation of fatty acids for a long term survival:

(a) Mean frequency with SEM of viable (DAPI-) CD4+ T cells stimulated once (white bar) or repeatedly (black) following restimulation with α-CD3/α-CD28 antibodies in the presence of the glycolysis inhibitor 2-DG (2 mM) for 72h relative to cells cultivated without inhibitor as determined by flow cytometry. **p=0.01, Mann Whitney two tailed test, experiment was repeated 3 times (b) absolute mean cell number (error bar = SEM) of *in vitro* CD3/CD28 repeatedly stimulated Th1 cells in control full nutrient medium (black line) or medium supplemented with 150 µM Etomoxir (red line). Surviving cells were counted by CD4+/PI-gating strategy in FACS (c) % of extracellular acidification rate derived from added glucose in presence of 150 µM Etomoxir before and after stimulation with PMA/Ionomycin (indicated with an arrow). Experiment was repeated 3 times.

On day 2 and day 3 after anti-CD3/ anti-CD28 stimulation cell numbers were higher in Etomoxir treated repeatedly stimulated Th1 cells, nevertheless did not survive 5 days post stimulation (Figure 2-4b). Analysis of glycolytic rate using Seahorse showed that Etomoxir treatment increases glucose - derived glycolytic rates in repeatedly stimulated T cell (Figure 2-4c). PD-1, marker of follicular T helper cells has been previously linked to an increase of oxidation of fatty acids and a decrease of glycolysis in T cells (Patsoukis et al. 2015) and it is also a marker of pathogenic synovial T cell population found in the joint of RA patients (Rao et al. 2017a). We observed upregulated mRNA expression of *PD-1* in *in vitro* three-time stimulated murine Th1 cells, compared to once stimulated murine Th1 cells (Figure 2-5).

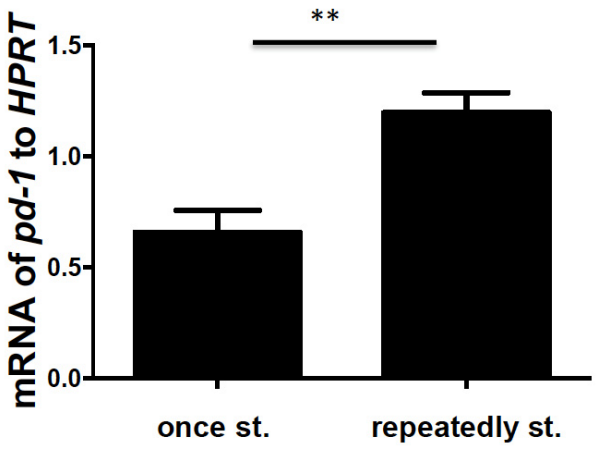

Figure 2-5 Three times stimulated Th1 cells upregulate *pd-1* mRNA expression:

Prior to the RNA isolation and measurement of real-time expression of *pd-1*, once and repeatedly (three times) stimulated Th1 cells were cultured *in vitro* for 6 and 18 days, respectively, and re-stimulated by PMA/ Ionomycin for 6 hours. Mean with SEm is indicatedTwo tailed t-test, 0.0083. n=2

2.2 Phenotypical analysis of *ex vivo* isolated Juvenile idiopathic Arthritis CD4+ T cells

In vitro murine culture studies suggested survival dependency of repeatedly stimulated Th1 cells on fatty acid oxidation. Therefore, we aimed to relate findings of *in vitro* culture model to metabolic phenotype of *ex vivo* autoimmune CD14- CD4+ T cells that were isolated from a synovial fluid of patients with rheumatoid disease represented by Juvenile idiopathic Arthritis (JIA). First, to exclude macrophages and monocytes that express CD4 surface marker, CD14+ cells were isolated out of cellular suspension by Magnetic Activated Cell Sorting (MACS). Subsequently, CD4+ cells were isolated and analysed by flow cytometry for memory markers, cytokine and transcription factor expression. The phenotypic characterization of CD4+ T cells has shown that 92% of CD4 T cells from synovial fluid were memory cells (Figure 2-6). On contrary, blood cells were shown to contain about 52% of CD45 RO+ cells and 38% of CD45 RO- CD45 RA+ CD4 T cells. In further experiments we will consider cells isolated from JIA patients as prevalently CD45RO+ positive.

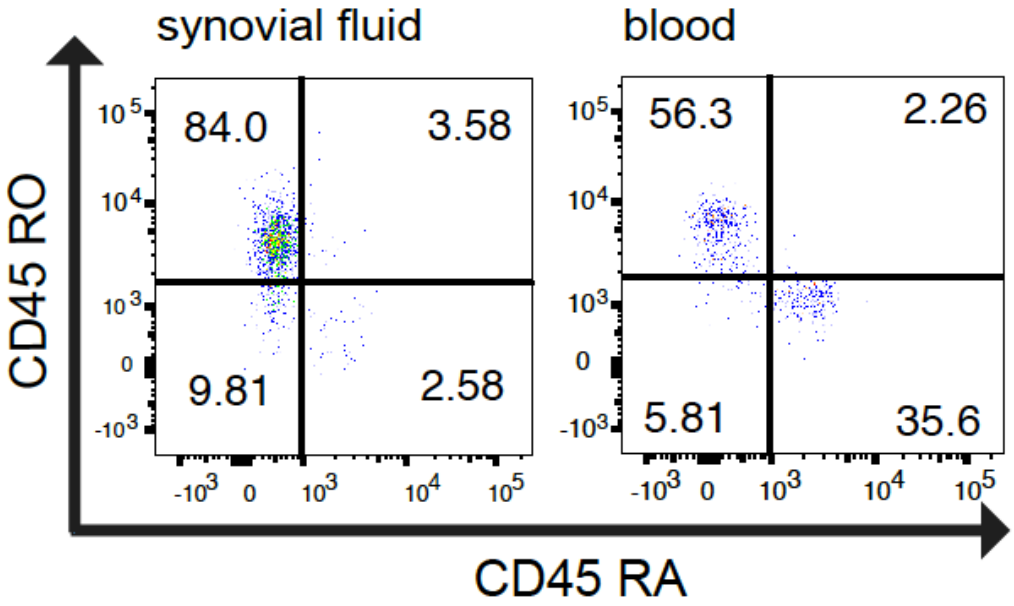

Figure 2-6 *Ex vivo* isolated CD4+ CD14- T cells from joints of JIA patients are memory CD4+ T cells:

Flow cytometric analysis indicating frequencies of CD45RA+ naive and CD45RO+ memory phenotype cells among CD3+CD4+ T cells in blood and synovial fluid of JIA patients

In order to further characterize cells by cytokine expression, cells were re-stimulated for 5-7 hours in complete human RPMI medium with PMA/ Ionomycin and BrefeldinA, fixed, stained for lineage-specific cytokines and analyzed by flow cytometry. Upon PMA/Ionomycin re-stimulation, in average 52% of cells expressed IFN-γ and approximately 70% of CD4 T cells expressed TNF-α, Th1- associated cytokines (**Figure 2-7**). Only minimum of cells expressed IL17 (**Figure 2-7**a,b). We found a low number of IL-4 and IL-21 expressing cells while IL-10 was expressed on average by 8% of cells, and these cells were also producers of IFN- γ (Figure 2-7b). Majority of T cells isolated from synovial fluid also expressed T-bet in contrast to effector and memory T cells isolated from patients' blood (Figure 2-7c).

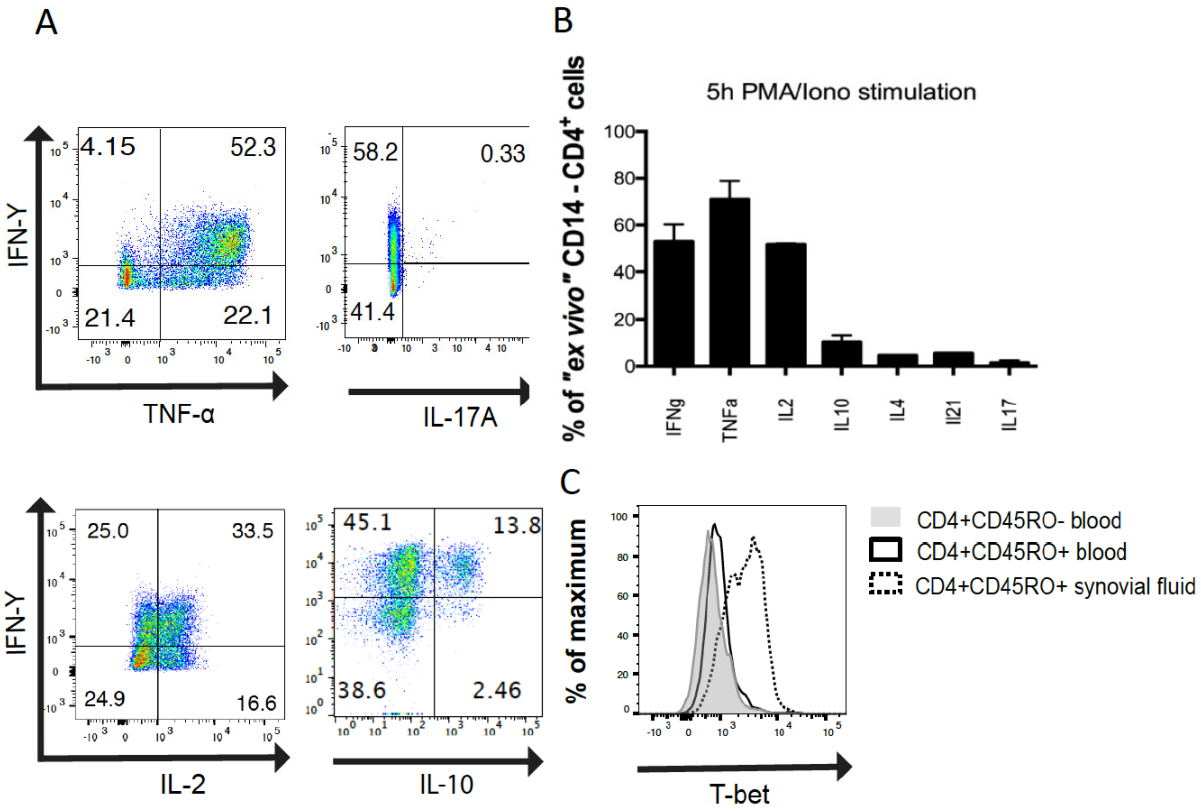

Figure 2-7: *ex vivo* isolated CD4+ CD14- T cells from joints of JIA patients secrete upon re-stimulation Th1- specific cytokines and lineage specific transcription factor T-bet:

(A) Expression of IFN-γ, TNF-α, IL-2, IL-10, IL-4, IL-21 and IL-17A in *ex vivo* isolated synovial T cells as analyzed by intracellular cytokine staining following restimulation with PMA/Ionomycin for 5-7 hours. A representative plot of n=5 is shown. (B) Frequencies of synovial fluid CD4+ T cells expressing IFN-γ, TNF-α, IL-2, IL-10, IL-4, IL-21 and IL-17A. Data shown are mean+/-SEM of 5 patients. (C) Flow cytometric analysis of T-bet expression in CD4+CD45RO- T cells from blood and CD4+CD45RO- and CD4+CD45RO+ T cells from synovial fluid of a JIA patient. A representative staining of n=3 is shown.

PD1+ CD4+ T cells were shown to be expressed in the synovial fluid of rheumatoid arthritis patients (Li et al. 2014). Recently it was suggested that peripheral CD4+ T cell population of cells expressing PD1 play a pathogenic role in synovial inflammation (Rao et al. 2017a). To address expression of PD1 in memory CD4+ T cells isolated from synovial fluid of JIA patients, we measured PD1 expression in CD4+CD45 RO+ cells acquired from their synovial fluid and blood. Upfront, CD15+, dead and CD14+ cells and gated out using flow cytometry.

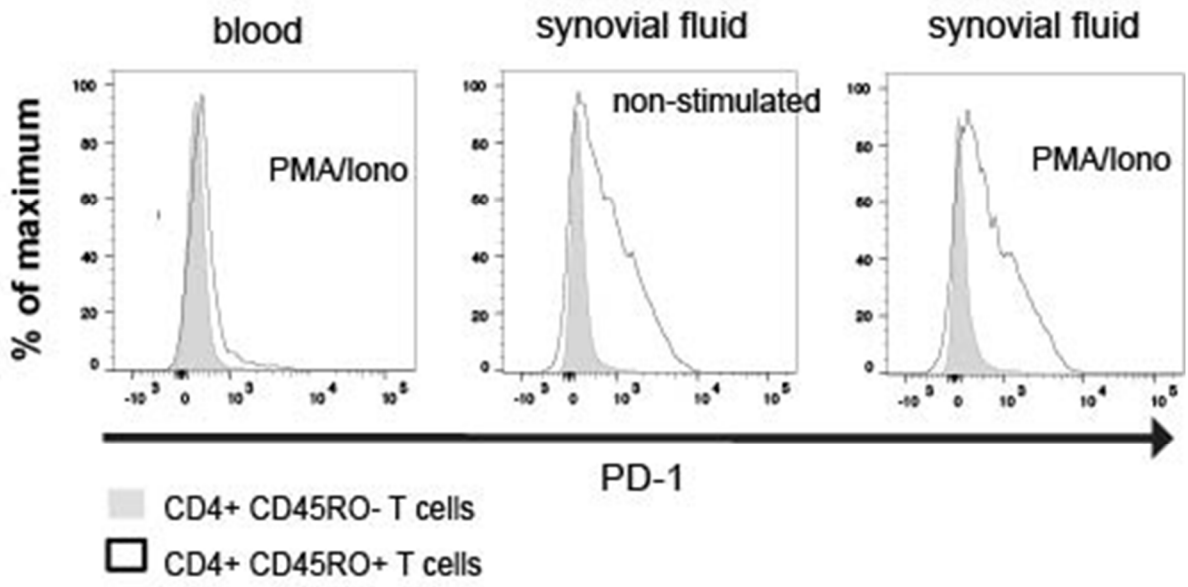

Figure 2-8: *ex vivo* isolated memory CD4+ T cells from synovial fluid of JIA patients express PD1+ in contrast to the memory CD4+ T cells isolated from the blood:

Mean fluorescence intensity (MFI) of PD1 staining in CD14- CD15 - alive effector (CD4+CD45 RO-) and memory (CD4+CD45 RO+) T cells isolated from blood and synovial fluid that were re-stimulated with PMA and Ionomycin. PD-1 staining of non-stimulated CD14- CD15 - CD4+CD45 RO+ and CD4+CD45 RO- cells is also shown (synovial non-stimulated). Staining was repeated three times.

To investigate the influence of individual metabolic pathways on survival of stimulated or non-stimulated CD14-CD4+ T cells isolated *ex vivo* from patients with Juvenile idiopathic arthritis, we employed selective inhibitors of glycolysis - 2DG, glutaminolysis - DON (Willis and Seegmiller 1977), fatty acid oxidation - Etomoxir and OXPHOS – Oligomycin (Bertina et al. 1974) and compared CD4+ synovial cells to a CD4+cells isolated from blood of these patients (Figure 2-9).

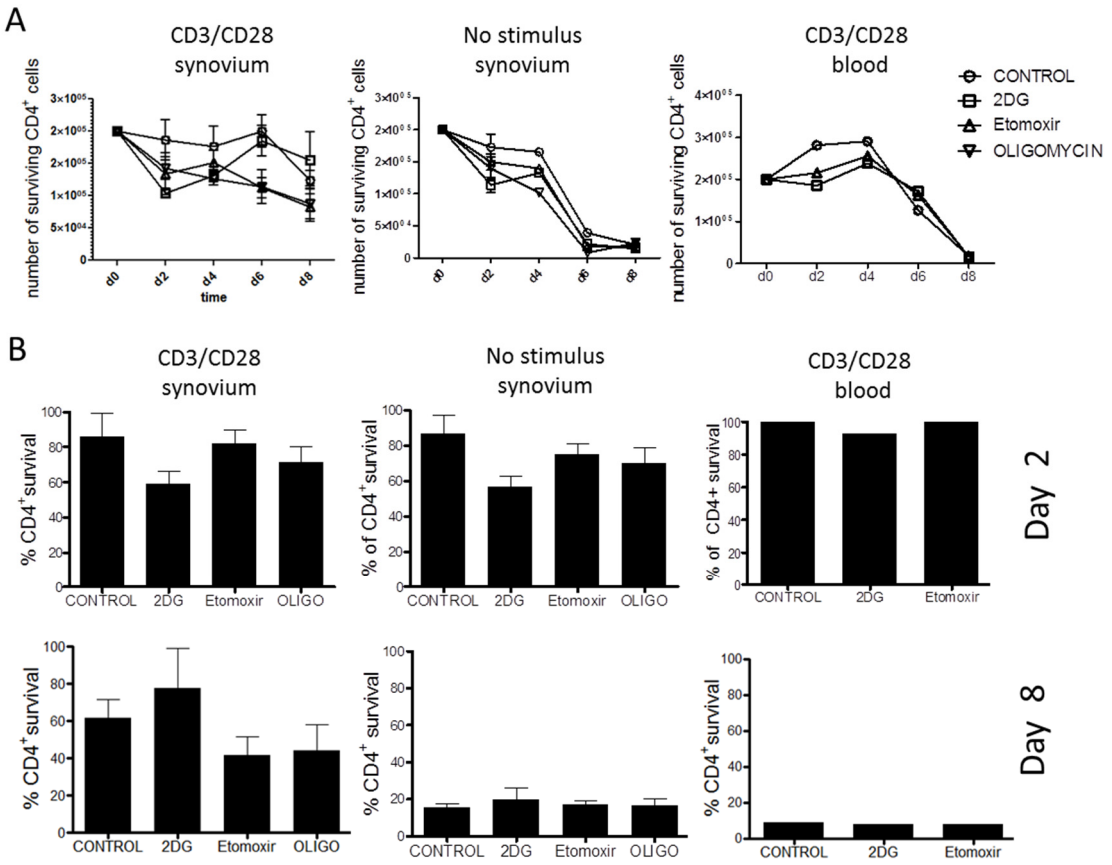

Figure 2-9: Selective sensitivity of JIA rheumatoid synovial CD4+ T cells to inihibitors of oxidative metabolism:

CD4+ CD14- T cells were isolated from joints of rheumatic patients using magnetic sorting and cultivated with human serum in 4 % oxygen with addition of inhibitors of glycolysis (2DG) fatty acid oxidation (Etomoxir) or OXPHOS (Oligomycin) in presence or absence of anti-CD3/anti-CD28 (a) mean total cell count (with SEM) with of alive (DAPI-) CD4+ number of cells isolated from synovium and blood, stimulated with anti-CD3/ anti-CD28 or synovial cells not stimulated over course of 8 days (b) mean % (with SEM) of surviving of stimulated, unstimulated synovial and blood derived CD4+ cells on day 2 and day 8 after initiation *ex vivo* culture. n= 2. Differences were not significant.

Upon stimulation with anti-CD3/ anti- CD28, synovial CD4+ T cells treated with all metabolic inhibitors were able to survive until day 8 (Figure 2-9a), further time points were not assessed. Surprisingly, if cells were not stimulated, cells were unable to survive after day 4 (Figure 2-9a – no stimulus, synovium). Cells isolated from blood stimulated with anti - CD3/ anti-CD28 were not able to survive more than 4 days in the culture (Figure 2-9a), irrespective of the metabolic inhibition (Figure 2-9a- CD3/CD28, blood). Two days after *ex vivo* isolation both anti-CD3/ anti-CD28 stimulated and un-stimulated synovial CD4+ CD14- T cells treated with glycolysis inhibitor 2DG had on average 20% lower survival than control cells (Figure 2-9b). Cells treated with inhibitor of oxidative phosphorylation, Oligomycin, presented on

average 12% less surviving cells than control on day 2. On day 8, non-stimulated and blood derived CD4 memory cells did not survive, while 60% of synovial CD4 T cells cultured in control medium survived up until day 8, compared to 40% of cells treated with inhibited OXPHOS or FAO and compared to 78% of cells treated with glycolysis inhibitor 2DG (Figure 2-9b).

2.3 Engagement of TCR is not crucial for homeostatic proliferation of *ex vivo* isolated CD4 T cells

Next, we investigated proliferation of CD4+ T cells isolated *ex vivo* from patients with Juvenile Idiopathic Arthritis in control medium and in medium containing metabolic inhibitors. Cells were stained with proliferation dye and measured in 2 day intervals with flow cytometry. Proliferation was comparable between stimulated (stim.) and unstimulated (ns.) CD4+ T cells (Figure 2-10a). As assessed by measurement of proliferation of surviving cells on day 8, cells proliferated minimally (**Figure 2-10**a). Metabolic inhibition of OXPHOS by Oligomycin, FAO by Etomoxir or glycolysis by 2DG had comparable effect on proliferation that was slightly hampered in comparison to control conditions (Figure 2-10b).

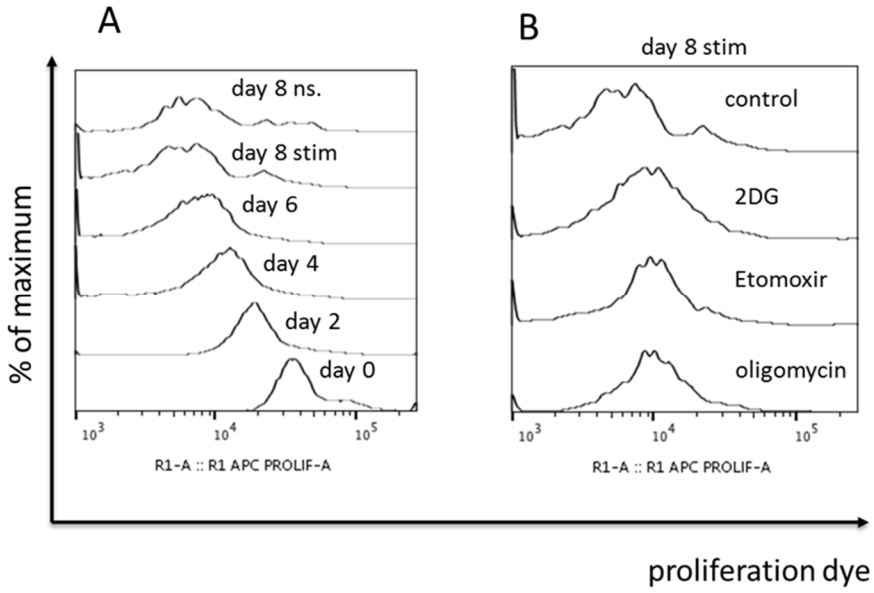

Figure 2-10 Proliferation of *ex vivo* cultured anti-CD3/anti-CD28 stimulated and not stimulated CD4+ CD14- cells isolated from JIA patients:

(a) Cells cultured in control medium assessed by staining with Cell Proliferation Dye eFluor 670 by flow cytometry (b) proliferation of cells cultured in media containing metabolic inhibitors of glycolysis (2mM 2DG) fatty acid oxidation (150 µM Etomoxir) or OXPHOS (2 µM Oligomycin).

2.4 Pathogenic memory PD1 + CXCR5- synovial CD4+ T cells is selectively sensitive to inhibition of fatty acid oxidation

Studies suggest that the expanded CD4+ PD1 + CXCR5- population activates antibody production in B cells at the inflamed joint and therefore supports ongoing autoimmune inflammation (Rao et al. 2017a).

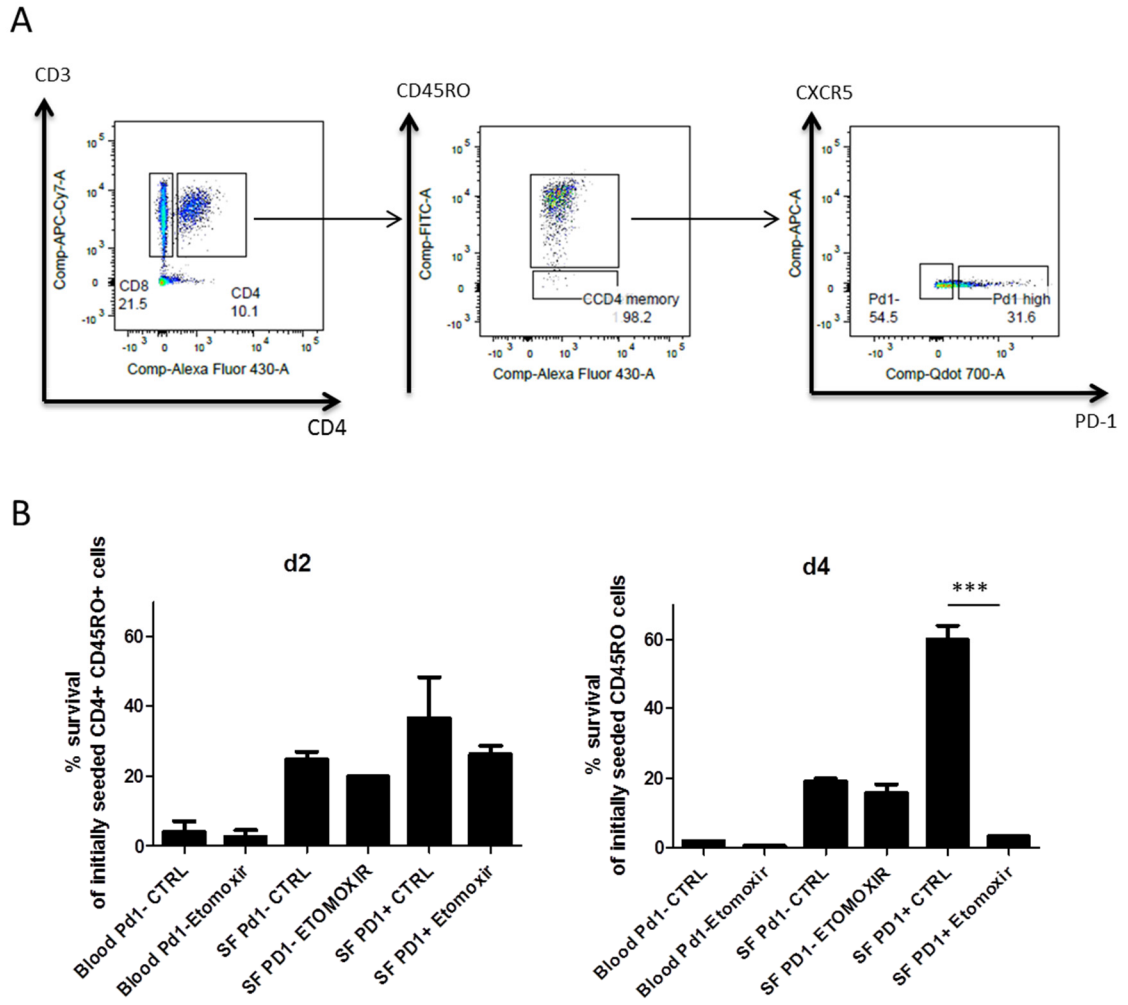

Figure 2-11 Memory CD4+ CD45RO+ PD1+ T cells isolated from inflamed tissue of JIA patients do not survive in FAO limiting condition.

(a) CD4+ CD14- CD45RO+T cells were sorted according to a PD1 and CXCR5 expression, seeded in density of 70 000 cells and stimulated by CD3/CD8 RPMI+10% human serum+ 200 µM Etomoxir (FAO blocker)(b) % survival of initially seeded cells was assessed on day 2 and day 4 after anti-CD3/anti-CD28 stimulation. Cells were cultured under 4 % oxygen. Statistics: two- tailed T test, P value= 0.0002 (***)

PD-1 inhibits PI3K mediated downregulation of CPT1, resulting in preference to oxidize fatty acids as an energy source over glycolysis (Patsoukis et al. 2015). Selective dependence of pathogenic memory CD4+ T cells present in rheumatic synovium on fatty acid oxidation have not been shown so far. We isolated CD14- CD4+ T cells from blood and synovial fluid of Juvenile Idiopathic Arthritis patients by magnetic sorting and used flow cytometry to sort CD15 - CD45RO+ CXCR5- PD1+ and CD15- CD4+ CD45RO+ CXCR5- PD1- populations of T cells. Subsequently, we investigated whether this pathogenic PD1 expressing population of synovial CD4+ T cells within the synovial memory T cells population can be selectively starved by inhibition of fatty acid oxidation *in vitro*. Sorted populations were cultured *ex vivo* in medium with or without fatty acid oxidation inhibitor Etomoxir. Blood CD4+ CD45RO+ T cells did not survive culture *in vitro* under control or fatty acid oxidation inhibitory conditions, and PD1- cells had comparable low survival rate in both control and fatty acid oxidation limiting conditions on day 4, PD1+ memory CD4+ T cells cultured in control medium had significant survival advantage over PD1– memory T cells. This survival advantage could be abrogated by inhibition of fatty acid oxidation, as on day 4 cells treated with Etomoxir had lover survival rate than cells not expressing PD1, treated with Etomoxir (Figure 2-11b).

2.5 CD4+ CD45RO + PD1+ CXCR5- pathogenic memory T cells upregulate transcription factor *Twist1* that mediates downregulation of glycolytic and oxidative pathways

Next, we were interested in further characterization of pathogenic PD1+ CXCR5 – CD4+ T cell population. To this end we measured mRNA of hallmark transcription factor of autoimmune cells in rheumatoid arthritis, *Twist1*. Cells were sorted by flow cytometry, left unstimulated, re-suspended in a RNA lysis buffer and RNA was isolated. *Twist1* expression was assessed by real time PCR. CD4+ CD45RO + PD1+ CXCR5- pathogenic memory T cells had increased transcriptional levels of *Twist1* (Figure 2-12) in contrast to CD4+ CD45RO + PD1- CXCR5- or blood counterparts.

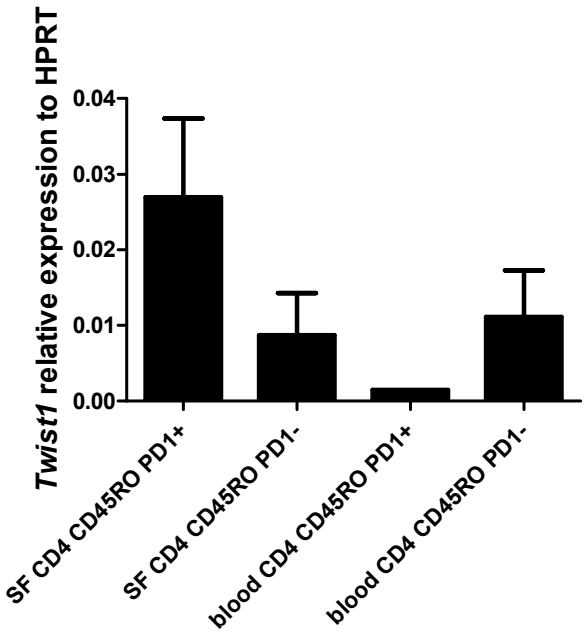

Figure 2-12 mRNA of *Twist1* is upregulated in PD1+ subset of CD4 + CD45 RO+ cells isolated from synovia of Juvenile Idiopathic Arthritis patients:

Twist1 mRNA expression in ex vivo isolated PD-1+ and PD-1- CD4+CD45 RO+ cells isolated from synovial fluid (SF) or blood of JIA patients. Cells were sorted by FACS. mRNA levels are shown relative to *HPRT*. n=2 mean with SEM. Significance was not determined due to a limited amount of sample.

Twist1 has been implicated in regulating the survival of Th1 cells (Niesner et al. 2008; Haftmann et al. 2015), however its function in energy homeostasis and fatty acid metabolism is only known in adipocytes (Pan et al. 2009; Pettersson et al. 2010) and muscle cells (Mudry et al. 2015b). To investigate impact of Twist1 on metabolism of once and repeatedly activated Th1 cells, we measured lactate production and oxidative consumption in WT and *Twist1*-deficient cells of both phenotypes. Recombinant mouse used harbours Cre recombinase, driven by CD4 promoter. Cre recombinase recognizes two LoxP sites that are flanking Twist1 allele and therefore Twist1 is only deleted in CD4 T cells.

Twist1 deficiency resulted in an increased ECAR both in once (Figure 2-13a) and in three-times activated (Figure 2-13b) Th1 cells. Increased glycolysis was observed after addition of glucose also in three times stimulated Twist1 deficient Th17 cells, nevertheless the difference in glycolysis between wild type and Twist1 $^{fl/fl}$ deficient repeatedly activated Th17 was not after re-activation with PMA/Ionomycin (Figure 2-13c). Surprisingly, OXPHOS was downregulated by Twist1 during activation with PMA/Ionomycin in three time stimulated Th1 cells (Figure 2-13e) in a higher level than in once (Figure 2-13d) stimulated Th1 cells. Twist1

deficiency did not influence oxidative consumption of three time stimulated Th17 cells (Figure 2-13f). Legend to the **Figure 2-13** on the next page.

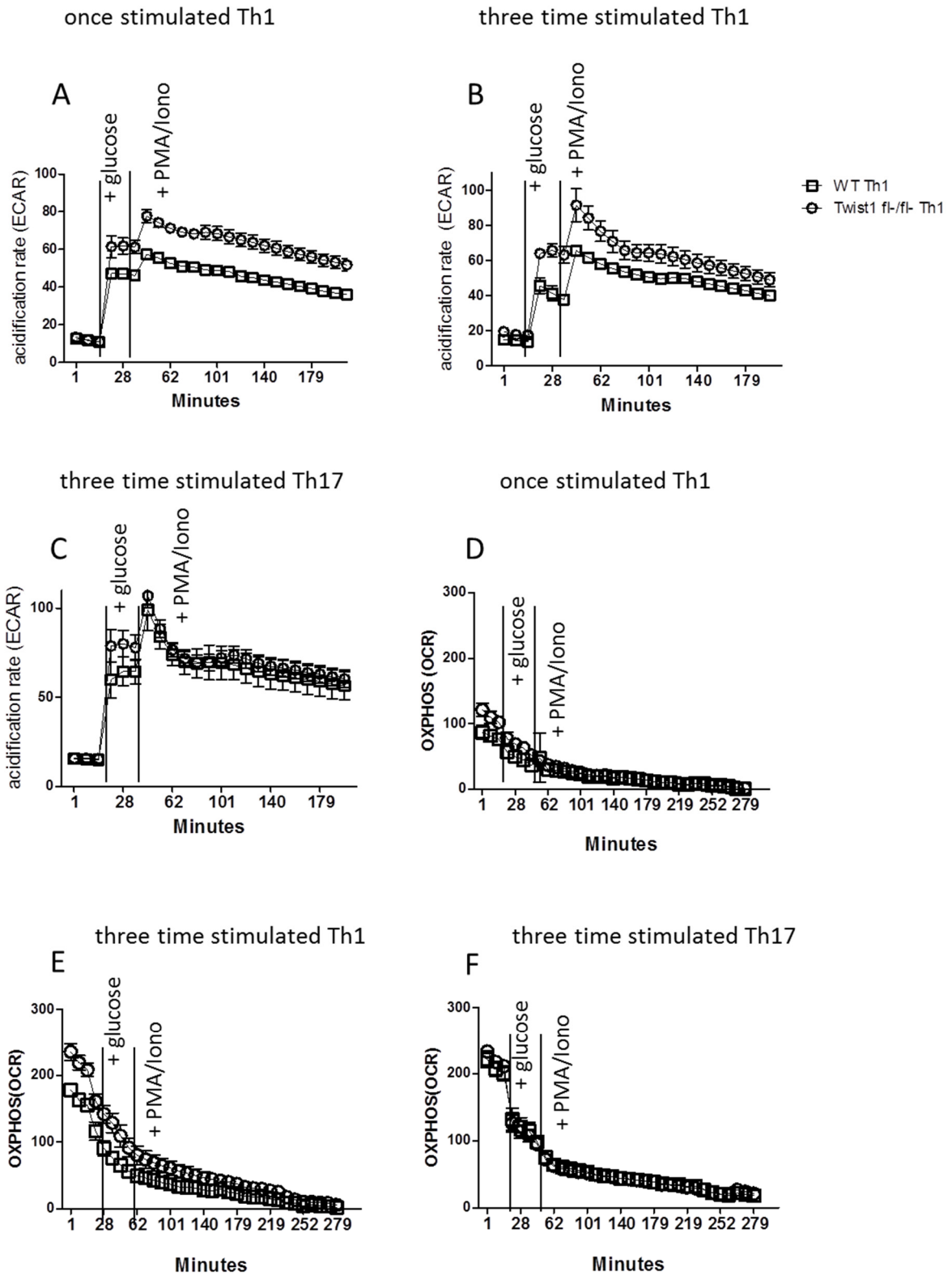

Figure 2-13 Twist1 inhibits metabolism upon activation in once and repeatedly stimulated Th1 cells:

(a,b,c) Extracellular acidification rate as indicator of glycolysis (ECAR) and (d,e,f) oxidative consumption rate (OCR) as an indicator of oxidative phosphorylation of Twist fl/fl CD4 Cre Th1 cells (circle) and Twist wt/wt CD4 Cre cells (square) stimulated with OVA peptide *in vitro* for (a,d) 6 days (once stimulated Th1 cells) or (b,e) 18 days (three time stimulated) and (c,f) three time stimulated Th17 cells. Significance for a) p= 0,0006 at 28 min; p=0.0001 at 52 min, b) p=0.0001 at 28 min; p=0.0013 at 52 min c) p=0.0135 at 28 min; p=ns at 52 min d) p=0.0027 at 28 min; p=0.0111 at 52 min e) p=0.0002 at 28 min; p=0.051 at 52 min. f) p=0,36 (ns) at 28 min; p=0.47 (ns) at 52 min, all paired t test. Graphs are representative of three independent experiments.

2.6 Global Affymetrix transcriptome of repeatedly stimulated Th1 Twist1 fl/fl cells

To understand Twist1-mediated regulation of metabolic genes in chronically stimulated T cells, we performed comparative Affymetrix analysis of repeatedly stimulated *Twist1*-deficient cells. Twist1 was deleted by recombinant protein TAT-Cre 3 days before last re-activation for RNA collection and compared to repeatedly stimulated, TAT-Cre treated WT Th1 cells (Figure 3-1). In total, we observed differential regulation of 73 genes (Figure 2-15a). No Th1 cytokine coding genes showed up in the microarray analysis of repeatedly stimulated Th1 cells. This was surprising, as *Twist1* has been shown to downregulate Th1 cytokine expression *in vitro*: upon Twist1 overexpression in once stimulated Th1 cells, expression of IFN-γ, TNF-α and IL2 was decreased (Niesner et al. 2008). To validate this result, we analysed Th1 cytokine expression by flow cytometry in repeatedly stimulated Th1 cells. Twist1 WT once stimulated Th1 cells expressed in average 36% TNF-α /63% IFN-γ (Figure 2-14). In comparison to Twist1 deficient once stimulated Th1 expressed in average 55% TNF-α /83% IFN-γ, confirming previous findings of Twist1 mediated cytokine suppression. Twist1 WT *in vitro* repeatedly stimulated Th1 cells expressed in average 83% TNF-α /92% IFN-γ in comparison to Twist1 deficient repeatedly stimulated Th1 expressed in average 85% TNF-α /90% IFN-γ. Over all, in *in vitro* Th1 culture, Twist1 downregulate IFN-γ and TNF-α in once, but not in repeatedly stimulated Th1 cells.

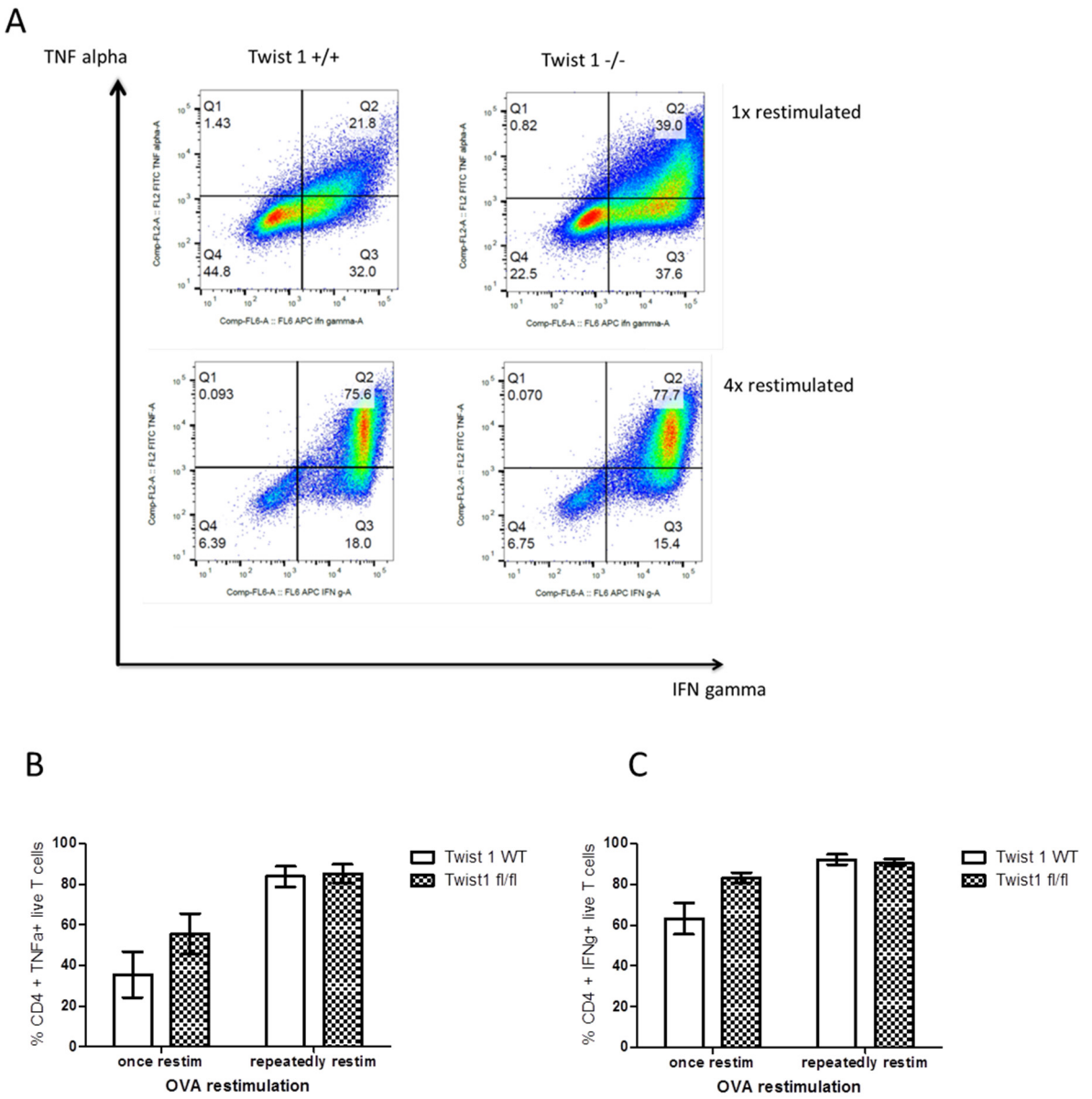

Figure 2-14 Twist1 downregulates IFN-γ, TNF-α in once, not repeatedly stimulated Th1 cells: Twist1+/+ and Twist1 fl/fl CD4 Cre OTII T_H1 cells were stimulated under T_H1-polarizing conditions (T_H1) with $ova_{327-339}$ and APCs for 6 days or 24 days. On d 6 surviving cells were isolated with Ficoll and restimulated with PMA/ ionomycin in the presence of brefeldin A for 5 h and stained for intracellular cytokine expression. Representative dot plots of cytokine expression in T_H cells are displayed. The single live cells were gated **(b)** frequencies of TNFα and **(c)** IFNγ cytokine expression. Data represent the mean ± s.d. of 5 independent measurements.

Interestingly, highest number of genes was involved in regulation of apoptosis and metabolism. Differentially regulated genes and their function in metabolism are shortly described here: *Ppp1r3b* codes for regulatory unit of protein phosphatase 1 (PP1), acting as

an activator (dephosphorylator) of glycogen synthase kinase (GSK3) and another enzymes involved in glycogen synthesis, glucose and triglyceride metabolism (Ceulemans and Bollen 2004; Zhang et al. 2014). In CD8 T cells, GSK3 inhibits T-bet function and upregulates PD1 expression (Taylor et al. 2016). Moreover, interactions between Twist1 and its transcriptional co-factors are controlled by GSK3 mediated phosphorylation (Lander et al. 2013). *Activating transcription factor 5* (*ATF5*) protein product promotes mitochondrial function and recovery from mitochondrial stress (Fiorese et al. 2016) and regulates lipid metabolism in adipocytes (Jiang et al. 2016). *Glut3* and *Glut1* are coding for proteins mediating a glucose uptake in T cells (Gerriets et al. 2015). *Oxct1* codes for an enzyme catalyzing formation of Acetoacetyl-CoA, critical metabolite for formation of cholesterol, lipid and short-chain acyl-CoAs (MacDonald et al. 2009). *PAICS (ADE2)* codes for Phospho - ribosylaminoimidazole- succino - carboxamide synthase involved in production of purines in a pentose phosphate pathway. *Gpx4* codes for a NAPDH dependent peroxidase, catalysing scavenging of lipid peroxides by oxidation of reduced glutathione. Upon inhibition of GPX4, cells undergo newly identified non-apoptotic iron dependent cell death called Ferroptosis (Yang et al. 2014; Yang and Stockwell 2016). *Rdh11* codes for a NADPH-dependent retinal reductase, playing a role in regulation of oxidative stress, and is also involved in the metabolism of lipid peroxide derived - short-chain aldehydes (Kasus-Jacobi et al. 2003). *Gpt2* codes for a mitochondrial alanine transaminase that catalyzes the reversible transamination between alanine and 2-oxoglutarate to generate pyruvate and glutamate during glutaminolysis, therefore being essential in TCA anaplerosis, crucial feature for synthesis of fatty acids (Ouyang et al. 2016).

Furthermore, we observed a differential expression of *Bnip3*. Bnip3 plays a role in autophagy-mediated survival of memory cells (Gang et al. 2015; O'Sullivan et al. 2015). Effector lymphocytes expressing Bnip3 elude cell death and do persist and form memory by utilizing lysosomal autophagy of accumulated mitochondria and ROS scavenging via activated AMPK- Atg3/Bnip3 pathway (O'Sullivan et al. 2015). Further, in liver, Bnip3 supports oxidation of fatty acids and in maintaining the mitochondrial integrity (Glick et al. 2012). Interestingly, we observed downregulation of transcription factor *Myc*, master regulator of glycolysis. Taken together, these gene transcriptional changes between wild type and *Twist1* knockout suggest the involvement of Twist1 in synthesis of fatty acids and protection of cells against reactive oxygen species, especially lipid peroxidation. Differential expression of Myc and Gpx4 has been confirmed by RT PCR (Figure 2-15c).

Transcriptional data are summarized in Table 4.

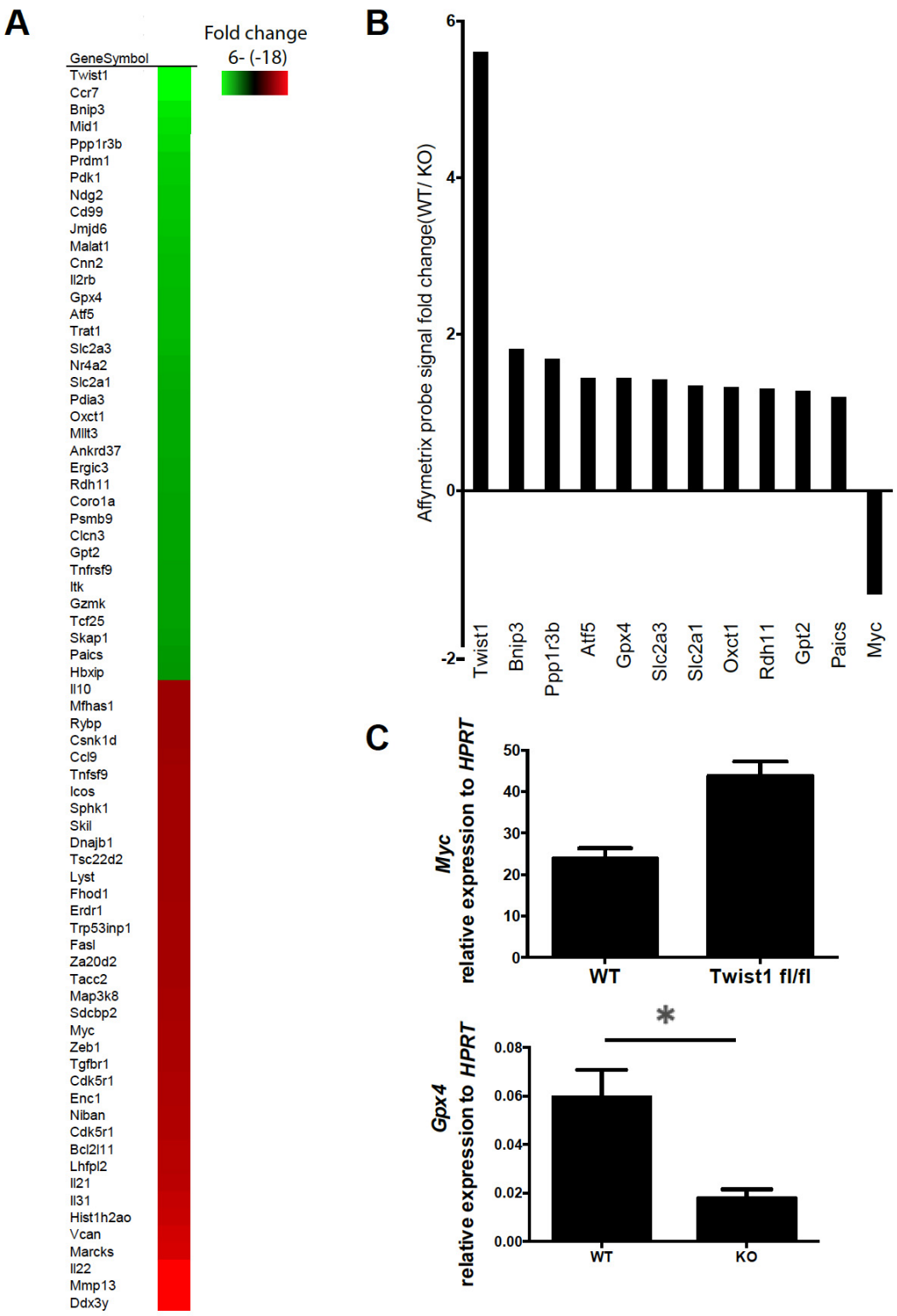

Figure 2-15: Affymetrix analysis of Twist1 mediated transcriptional expression (figure legend continues on the next page):

(a) Global heat transcriptome map of fold change between repeatedly stimulated Twist1 wild type CD4+ T cells (Twist1 wt/wt x OTII) compared to Twist1 deficient (Twist1 fl/fl x OTII). Green- genes transcriptionally upregulated by Twist1, red – genes are transcriptionally downregulated by Twist1 (b) metabolic signature of Twist1 depicted as ratio of Affymetrix probe signal measured in WT vs Twist1 fl/fl cells (c) RT PCR validation of transcriptional targets *Myc* and *Gpx4,* relative to HPRT.

2.7 Role of Twist1 in fatty acid metabolism

Next, we aimed to investigate phenotypic consequence of Twist1 deficiency on metabolism in repeatedly stimulated Th1 cells. We hypothesized that Twist1 deficient cells should have lover levels of *de novo* fatty acid synthesis during activation leading to the lower amount of intracellularly stored lipids. 24h after activation repeatedly stimulated cells had significantly higher lipid content than once stimulated cells (Figure 2-16a). Moreover, repeatedly stimulated Twist1 WT CD4+ T cells had higher lipid content than their Twist1 deficient counterparts, as observed by staining with Bodipy 505/515 dye (Figure 2-16a), however the difference was not significant (Mann Whitney test: P value= 0,0635). Memory cells were shown to be dependent on intracellularly synthesized lipids that are at the same time oxidized (Weinberg and Chandel 2014; O'Sullivan et al. 2014). Using metabolic inhibitors we investigated functional consequences of decreased lipid content in a repeatedly stimulated, Twist1 deficient Th1 cell culture. We tested whether *Twist1* deficient cells are capable of survival solely on fatty acids, we used specific inhibitor of glycolysis 2DG and concomitant inhibition of glutaminolysis by 6-diazo-5-oxo-L-norleucine (DON), excluding glutamine and glucose as energy sources. We observed selective death of three-time activated *Twist1*-deficient Th1 cells and not of three-times activated WT Th1 cells. Numbers of WT Th1 cells were maintained *in vitro* at least until day 9 (Figure 2-16b).

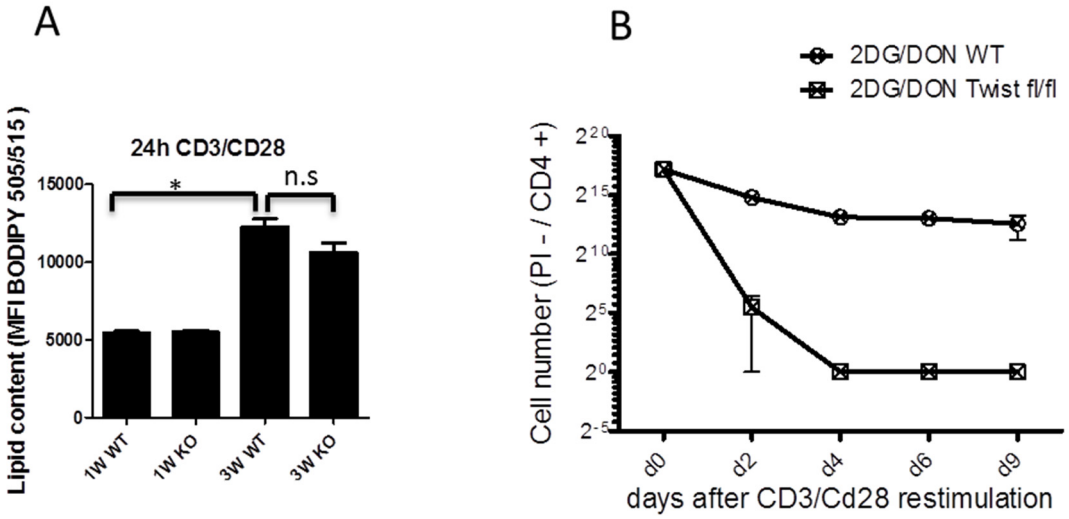

Figure 2-16: Twist1 mediates survival on fatty acid oxidation:

(a) Lipid content assessed by BODIPY 505/ 515 staining in once (1W) and repeatedly stimulated (3W) Twist1 wild type (WT) and Twist1 deficient (KO) cells. Statistics- MannWhitney test, P value *= 0,0159. (b) Absolute cell number of *in vitro* repeatedly stimulated Th1 cells treated with inhibitor of glycolysis (2DG) and concomitant inhibitor of glutaminolysis (DON). Surviving cells were counted by CD4+/PI- gating strategy in FACS. Significance could not be determined.

2.8 Long term survival is impaired in *Twist1* deficient cells upon oxidative stress induced by Etomoxir

Differential regulation of several genes involved in oxidative stress protection led us to investigate the response of *Twist1* deficient cells to the oxidative stress induced by metabolic inhibition of FAO by Etomoxir, caused by depletion of NADPH (Estan et al. 2014; Pike et al. 2011). Using MitoSox Red and Bodipy C11 we measured ROS levels and Lipid peroxide levels, respectively, in Etomoxir, 2DG and DON treated cells.

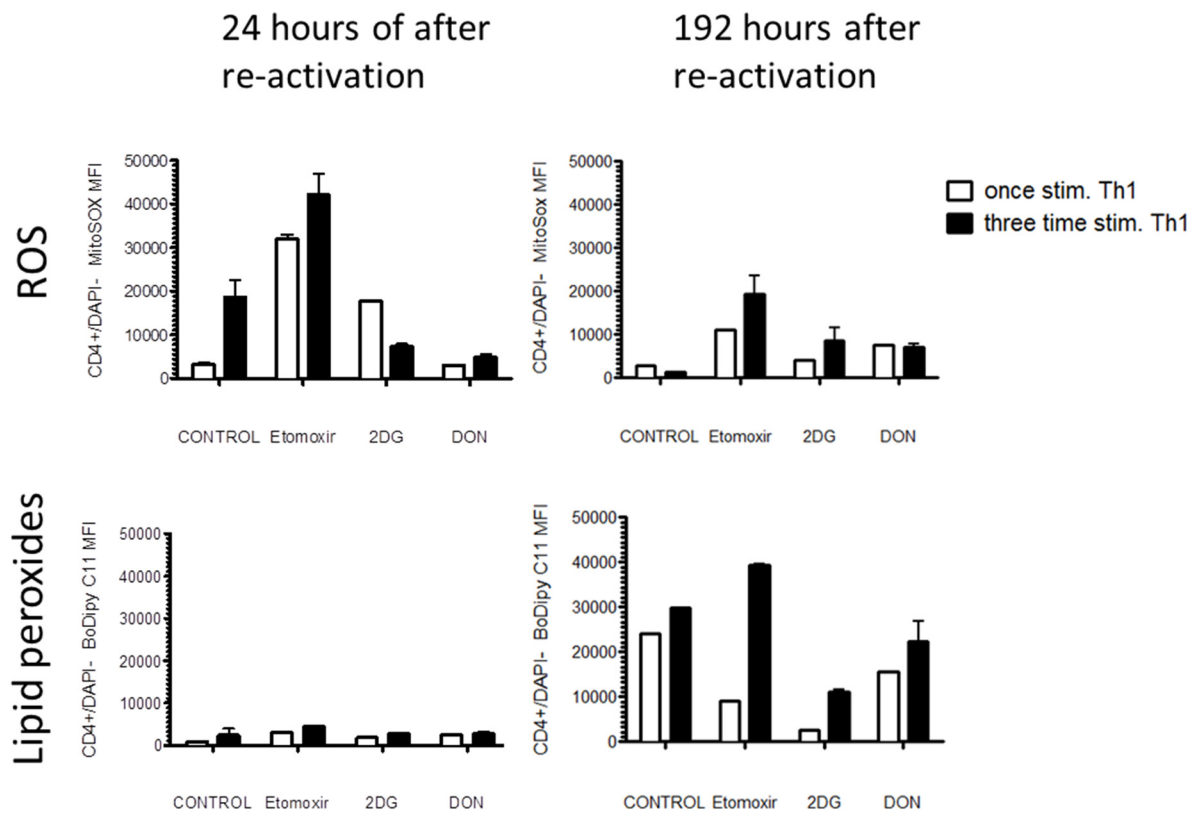

Figure 2-17: Repeatedly stimulated Th1 cells have higher level of ROS and lipid peroxides than once activated T cells:

Once and three times stimulated Th1 cells were re-stimulated with CD3/CD28 in the absence (CONTROL) or presence of metabolic inhibitor of fatty acid oxidation (Etomoxir), glycolysis (2DG) and glutaminolysis (DON). Levels of ROS and lipid peroxidation were measured by flow cytometry employing staining with fluorogenic dyes MitoSox Red and Bodipy C11, respectively, 24 and 192 hours after CD3/ CD28 re-activation. Significance was not determined due to a low number of replicates.

In comparison to other metabolic inhibitors, Etomoxir induced increased levels of mitochondrial superoxides in both, once and repeatedly stimulated Th1 cells 24 hours after CD3/CD28 re-activation (Figure 2-17). While ROS levels did decrease in a contraction phase (192 hours after activation) Etomoxir treated - three times activated cells had markedly higher lipid peroxidation in comparison to once stimulated Th1 cells (Figure 2-17). Lipid peroxidation was increased in repeatedly stimulated, Etomoxir treated, Twist1-deficient Th1 cells on day 7 (168 h a.a.) in contrast to once stimulated, Etomoxir treated, Twist1 deficient Th1 cells (Figure 2-18).

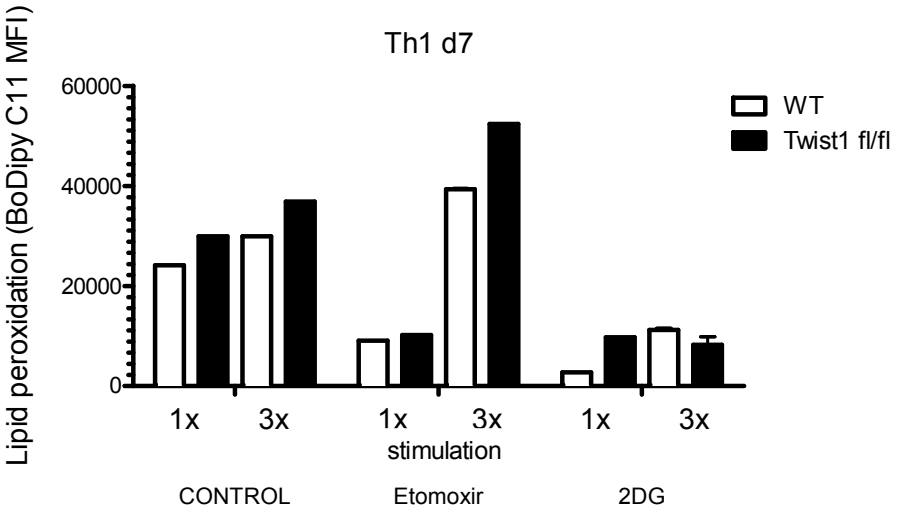

Figure 2-18: Etomoxir treated three time stimulated Twist1 deficient cells have high levels of lipid peroxidation on day 7 after re-activation with CD3/CD28: Accumulation of lipid peroxidation in WT and Twist1 deficient cells treated with selective inhibitors of FAO (Etomoxir) and glycolysis (2DG) once (1x) and repeatedly stimulated (3x) CD4+ Th1 cells determined by C11-BODIPY581/591 (2 µM) 7days after re-activation with CD3/CD28. Significance could not determined.

Next, we tested if Twist1 deficiency influences survival of once and repeatedly stimulated cells upon Etomoxir- induced oxidative stress. Survival of Twist1 deficient cells is shown as ratio of WT surviving cells to Twist1 deficient cells. 3 days after stimulation (d3), control medium treated once stimulated Twist1 deficient Th1 cells had survival advantage, assessed by cell number ratio of WT (wt/wt) to Twist1 deficient (fl/fl) cells (Figure 2-19). Etomoxir treated/Twist1 deficient/ once stimulated cells were affected by the treatment, nevertheless were able to recover - Twist1 deficient cells did survive similarly with or without treatment with Etomoxir 7 days after stimulation. Repeatedly activated Twist1 deficient Th1 cells cultured in control medium were comparable in survival to WT counterparts (ratio 1), but when Etomoxir was added to the culture, cells survived worst on day 3. Noticably, Etomoxir treatment affected long term survival of three times activated Th1 *Twist1* deficient cells, as the cell number was 2x lower than cells grown in control medium. We did not observe this effect in repeatedly stimulated Th17 cells (Figure 2-21).

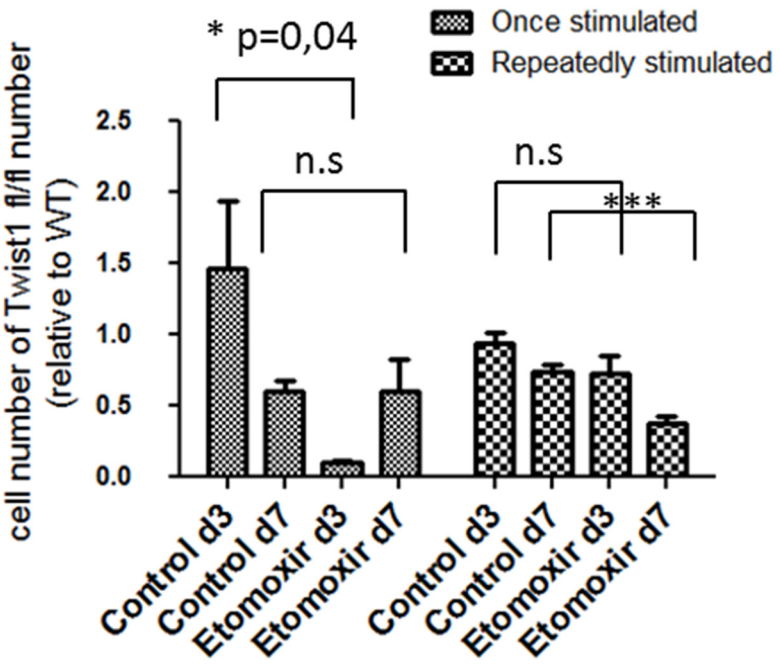

Figure 2-19: Three times stimulated Twist1 deficient cells treated with Etomoxir are deficient in a long term *in vitro* survival:

Twist fl/fl CD4 Cre OTII and Twist wt/wt CD4 Cre OTII cells stimulated once *in vitro* for 6 days (grey bars) or repeatedly (squared bars) 18d with OVA peptide, in a presence of splenic APCs under T_H1-polarizing conditions with medium control or medium containing 150 µM inhibitor of fatty acid oxidation Etomoxir. CD4+ PI- Cell number of Twist deficient cells is shown relative to WT control and was monitored over time by flow cytometer on day 3 and day 7

We were intrigued by deficiency in survival of Etomoxir treated once activated Th1 cell during activation phase, since fatty acid oxidation was not reported to be crucial for effector T cells survival during activation. Measurement of glycolysis rates in those cells showed that Etomoxir treatment diminished activation induced glycolysis completely in once stimulated WT and Twist1 deficient cells (Figure 2-20a) in contrast to control medium cells (Figure 2-13a). In contrast, chronically stimulated Th1 cells treated with Etomoxir were capable increase glycolytic rates (Figure 2-20b).

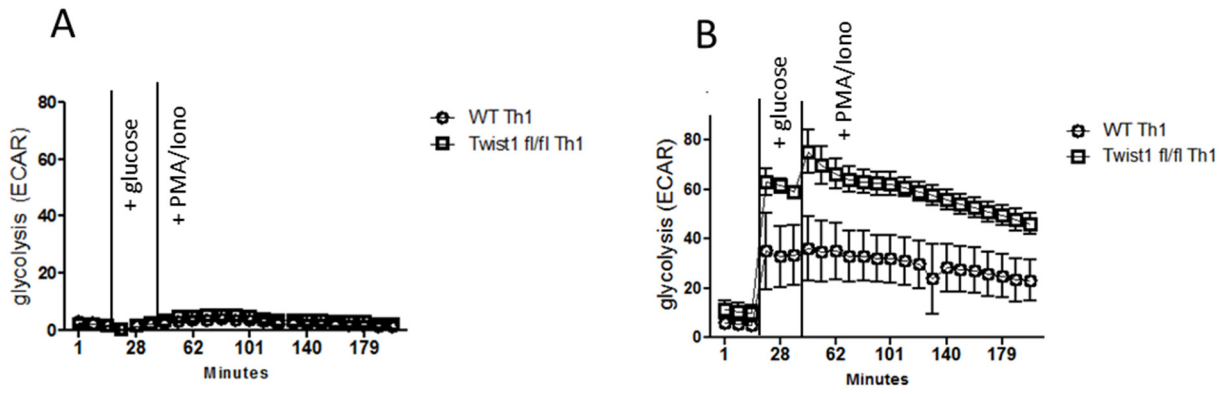

Figure 2-20: Upon treatment with Etomoxir, once stimulated Th1 cells are unable increase glycolysis upon addition of glucose and PMA/Ionomycin stimulation:

Acidification rates (ECAR) of CD4+ CD62L^{hi} OTII cells stimulated with OVA peptide under Th1 polarizing conditions *in vitro* for (a) 6 days (once stimulated) or (b) 18 days (repeatedly stimulated) days treated with 150 µM Etomoxir. Significance (a) 28 min = 0,4148 (n.s.), 52 min= 0,04 (*); (b) 28 min = 0,0052 (**), 52 min= 0,012 (*)

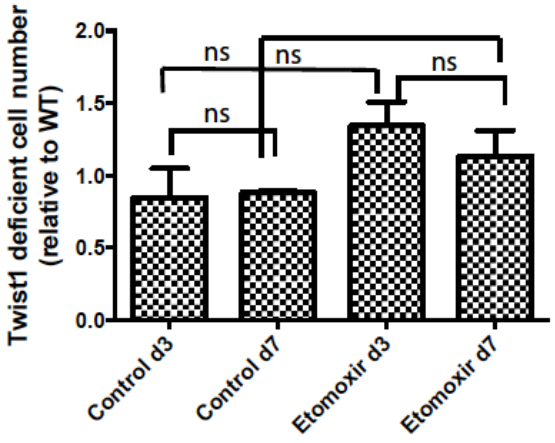

Figure 2-21 Three times stimulated Twist1 deficient Th17 cells are not sensitive to Etomoxir induced cell survival defect:

Cell number ratio of repeatedly stimulated Th17 Twist fl/fl CD4 Cre OTII to Twist wt/wt CD4 OTII CD4 Cre cells assessed on day 3 and day 7. Significance was determined by Mann Whitney test as follows.

3 Materials and methods

3.1 Materials

3.1.1 Mice

Chimeric conditional Twist1$^{fl/fl}$ mice was produced by Artemis Pharmaceuticals and crossed with Cre recombinase driven by CD4 promoter that mediates specific deletion of *Twist1* alleles in CD4 expressing cells. C57BL6/J mice used for APC isolation and OVA presentation were purchased from the Charles River Laboratories. To obtain mice with transgenic TCR, Twist1 $^{fl/fl}$ x CD4 Cre $^{+/-}$ and Twist1$^{wt/wt}$ x CD4 Cre$^{+/-}$ were crossed to OTII mice harbor a transgenic MHC class II restricted TCR responding to residues 323–339 of chicken ovalbumin. Twist$^{wt/wt}$ CD4-Cre$^{+/-}$ do not harbor loxP sites in the *Twist1* locus, thus express Twist1 and serve as wild type controls. OTII x Twist1$^{fl/fl}$ x CD4 Cre$^{+/-}$ and OTII x Twist1$^{wt/wt}$ x CD4 Cre$^{+/-}$, Twist1$^{fl/fl}$ x CD4 Cre$^{+/-}$ and Twist1$^{wt/wt}$ x CD4 Cre$^{+/-}$ mice were bred in the DRFZ animal facility. All mice were housed and bred under specific pathogen-free conditions in individually ventilated cages. Mice were handled in accordance with good animal practice as defined by the German animal welfare bodies. All experiments were approved by the federal state institution „Landesamt für Gesundheit und Soziales“(LAGeSo) in Berlin, Germany.

3.1.2 Human patient samples

The samples of peripheral blood and synovial fluid were collected from in total 11 patients by Dr. Anne Sae Lim von Stuckrad and Dr. med. Tilmann Kallinich. All children patients with Juvenile Idiopathic Arthritis sought medical attention for synovial inflammation at the Charité University hospital Berlin in the wake of a flare reaction with joint involvement. The medication was follows: 7 patients had no history of medication, 2 patients received 7,5 mg of MTX, one patient received Humira 40 mg every 4 weeks and one patient received Naproxen. The mean age of the JIA patients was 9,7 years with range 1-20 year. As part of standard relieve measures, synovial fluid was extracted from the knee joint by needle aspiration biopsy and collected in an uncoated syringe. Paired blood was collected simultaneously and cells were extracted using Ficoll density gradient technology.

3.1.3 Instruments

Name	Source
Cell culture hood Hera Safe	Kendro
Centrifuge 5810 R	Eppendorf
Centrifuge Heraeus Multifuge 3S-R	Thermo Scientific
BD FACSAria II	BD Biosciences
Forceps	Lab Laborfachhandel
Freezer -80 °C	Th. Geyer
Fridge 4 °C/ Freezer -20 °C Combi	Liebherr
Fridge 4 °C/ Freezer -20 °C Combi	Bosch
Ice machine	Ziegra
Bacterial incubator, CO_2 free	Heraeus B6
Hypoxia iGlove chamber	BioSpherix
Incubator C 150	Binder
StepOnePlus Real-Time PCR System	Thermo Scientific
MACS MultiStand	Miltenyi Biotec
MACSmix™Tube Rotator	Miltenyi Biotec
MACSQuant Analyzer	Miltenyi Biotec
Microcentrifuge Heraeus Fresco 17	Thermo Scientific
Microcentrifuge Heraeus Fresco 21	Thermo Scientific
MidiMACS™ Separator	Miltenyi Biotec
Nanodrop-1000	Thermo Scientific
Pipetboy	Integra
Pipetboy Easypet 3	Eppendorf
Pipette Research® Plus 10 µl	Eppendorf
Pipette Research® Plus 100 µl	Eppendorf
Pipette Research® Plus 1000 µl	Eppendorf
Pipette Research® Plus 2.5 µl	Eppendorf
Pipette Research® Plus Multichannel 300µl	Eppendorf

PowerPac™ HC power supply	Bio-Rad
QuadroMACS™ Separation Unit	Milentyi Biotec
Scissors	Fine Science Tools
Seahorse XF ^{e}96 Analyzer	Agilent Technologies
ThermoCycler	Biometra
Trans-Blot® Trubo™ Transfer System	Bio-Rad
Vortexer Analog Vortex Mixer	VWR
Vortexer MSI Minishaker	IKA
Water bath	Memmert
Zeiss LSM710	Carl Zeiss Microscopy

3.1.4 Software

Name	Source
Adobe Illustrator CC 2015	Adobe Systems
FlowJo Software version 9.9.4	FlowJo LLC
GraphPad Prism version 5.04	GraphPad Software
MACSQuantify™ Software version 2.6	Miltenyi Biotec
Microsoft Office Standart 2010	Microsoft
Seahorse Wave version 2.2.0.276	Seahorse Bioscience, Inc.

3.1.5 Disposable materials

Name	Source
0.5/1.5/2 ml reaction tubes	Eppendorf
0.2 ml Thin-walled Tubes with Flat Caps	Thermo Scientific
0.2 µm filter	Sarstedt
Cell culture plate 6-well	Greiner Bio
Cell culture plate 96-well	Greiner Bio
Cell strainer 70 µm	BD Biosciences
Cell strainer 100 µm	BD Biosciences
FACS tubes 5 ml	Carl Roth

Falcon tube 15 ml	Ibidi/ BD Biosciences
Falcon tube 50 ml	BD Biosciences
Filtered pipette tips 10 µl	Sarstedt
Filtered pipette tips 1000 µl	Sarstedt
Filtered pipette tips 20 µl	Sarstedt
Filtered pipette tips 200 µl	Sarstedt
Industrial mesh 70 µm pores	Sarstedt
Kimtech absorbent towels	Sarstedt
LS separation columns	Sefar Nitex
Pipette tips 10 µl	Greiner Bio
Pipette tips 1250 µl	Greiner Bio
Pipette tips 200 µl	Sarstedt
Pre-separation filter	Sarstedt
Reaction tube 0.5 ml	Sarstedt
Reaction tube 1.5 ml	Miltenyi Biotec
Reaction tube 2.0 ml	Sarstedt
Steritop filter	Sarstedt

3.1.6 Chemicals, reagents, antibodies for *in vitro* culture

Name	Source
2-Mercaptoethanol	Sigma-Aldrich
2-NBDG	Sigma-Aldrich
99,9% Ethanol	Roth
Etomoxir	Sigma-Aldrich
2DG	Sigma-Aldrich
DON	Sigma-Aldrich
Anti-mouse CD28	DRFZ
eCD3	Miltenyi
Anti-mCD4-FITC, clone: GK1.5	purified in DRFZ
Anti-hCD4-Cy5, clone: TT1	purified in DRFZ
Anti-CD4 MultiSort beads	Miltenyi

Anti-CD62L MultiSort beads	Miltenyi
Anti-CD62L- APC	purified in DRFZ
Anti-CD90.2 MultiSort Beads	Miltenyi
Anti-FITC MultiSort beads	Miltenyi
Anti-IFN , clone: AN18.1724	DRFZ
Anti-IL-4, clone: 11B11	DRFZ
Anti-HIF1 alpha (ChIP/Western Blot)	Abcam
Anti-HIF2 alpha (Western blot)	Novus Biologicals
BD Perm/Wash™ buffer	BD Biosciences
BODIPY 505/515	Thermo Fisher
BODIPY 581/591 C11	Thermo Fisher
Bovine Serum Albumin (BSA) Fraction V	PAN Biotech
Brefeldin A	BioLegend
Calibrant	Agilent Technologies (Seahorse Inc.)
Cell Proliferation Dye eFluor™ 670, eBioscience™	Thermo Fisher
cOmplete, EDTA-free	Roche Applied Science
cOmplete, Mini, EDTA-free	Roche Applied Science
Cytochalasin B	Sigma
Cytofix/Cytoperm buffer	BD Biosciences
DAPI	DAPI
dNTP Mix	Bioline
EDTA	Sigma-Aldrich
Ethanol, 100%	Carl Roth
Ficoll (Histopaque-1083)	Sigma
Ficoll Paque ™ PLUS	GE Healthcare
Fixable Viablility Dye eFluor 780	eBioscience
Fetal calf serum (FCS)	Biochrom
Formaldehyde solution, min 37%	Merck Millipore
GIBCO ® RPMI 1640 + GlutaMAX™	Life Technologies
GIBCO ® RPMI 1640 + GlutaMAX™ glucose	Life Technologies

free, with L-Glutamine	
Glycerol	Carl Roth
Glycine	Carl Roth
HISTOPAQUE®-1083	Sigma-Aldrich
IgG1 Fc, recombinant, human	R&D Systems
Interleukin 12, recombinant, murine	R&D Systems
Interleukin 2, recombinant, murine	R&D Systems
Interleukin 6, recombinant, murine	R&D Systems
Interleukin 23, recombinant, murine	R&D Systems
Human serum , male Ab plasma	Sigma
Ionomycin	Sigma
KCl	Carl Roth
L-Glutamine	Invitrogen
MgCl Stock 25mM – Tagman	Applied Biosystems
MgCl Stock 25mM – SybrGreen	Roche Diagnostics
MiroTracker® Red CMXRos	Thermo Scientific
Na2HPO4	Merck Millipore
Na3VO4	Sigma-Aldrich
NaF	Sigma-Aldrich
Penicillin/Streptomycin 100X	Life technologies
Peptide OVA $^{323-339}$	R.Volkmer-Engert, Humboldt University of Berlin
Perm/wash buffer 10x	BD
Poly-D-Lysine hydrobromide	Sigma- Aldrich
PMA	Sigma-Aldrich
Propidium Iodide	Sigma-Aldrich
RPMI 1640 + GlutaMAX-I	Life technologies
TGFβ	R&D Systems
XF96 Seahorse culture microplate	Agilent Technologies
XF96 four-port sensor cartridge	Agilent Technologies

3.1.7 List of antibodies used for flow cytometry

Name	Clone	Fluorochrome	Source
Anti- Mouse CD4	GK 1.5	FITC	DRFZ
Anti-Mouse TNF-α	MP6-XT22	PerCP	DRFZ
Anti-Mouse IFN-γ	XMG 1.2	PerCP.Cy5.5	eBioscience
Anti- Mouse IL-2	JES 65H4	eFl450	eBioscience
Anti- Mouse IL-17A	TC11-18H10.1	FITC	Biolegend
Anti- Mouse IL-17F	eBio18F10	Alexa Fluor® 647	eBioscience
Anti- Mouse IL10	JES 516E3	PE	eBioscience
Anti human CD14	TM1	PE	DRFZ
Anti human CD3	SK7	APC Cy7	eBioscience
Anti human CD4	Okt4	BV 510	Biolegend
Anti human CD4	SK3	BV421	BD
Anti human CD4	RM4-5	PE-Cy7	eBioscience
Anti human CD45RO	Uchl1	FITC	DRFZ
Anti human CD45RA	4G11	APC	DRFZ
Anti human CXCR5	clone	APC	Milteney
anti human IL-2	MQ117H12	BV 605	Biolegend
anti human IL-4	8D48	PE	Biolegend
anti human IL-21	7H20-I19-M3	Biotin	Biolegend
Anti human IFN-γ	4SB3	Pe- Cy7	Biolegend
Anti human TNF-α	cA2	APC	Milteney
Anti human IL-17 A	64Dec17	Al 488	eBioscience
Anti human IL-17 A	Bl168	APC- Cy7	Biolegend
Anti human T-bet	4B10	APC	Biolegend
Anti human IL-10	13099223	PE	Milteney

3.1.8 Buffers and Media

Buffers (reagent)	Final concentration / amount
PBS buffer	In house
NaCl	137 mM
KCl	2.7 mM
Na2HPO4	10 mM
KH2PO4	1,8 mM
HCl	Add until pH = 7.4
PBS/BSA	In house
PBS	500 ml
BSA	1 g
PBS/BSA/EDTA	In house
PBS/BSA	500 ml
EDTA	2mM
Media	Component concentration/ company
RPMI complete mouse	
GIBCO ® RPMI 1640 + GlutaMAXTM	500ml
FCS	5ml
2-Mercaptoethanol 50 mM	500 µl
Penicillin/Streptomycin	5ml
RPMI complete human	
GIBCO ® RPMI 1640 + GlutaMAXTM (500ml)	500ml
Human male Ab serum	5%
Penicillin/Streptomycin (5ml)	1%

3.1.9 Commercial Kits

Kit name	Source
Direct-zol RNA kit	Zymo research

LightCycler® FastStart DNA Master SYBR Green	Roche
RNeasy Mini Kit	Quiagen
Taqman Reverse Transcription Reagent Kit	Life technologies

3.2 Methods

3.2.1 Isolation of primary naïve mouse $CD4^+$ T cells

To start a culture of once and repeatedly stimulated T helper 1 cells, spleens and peripheral lymph nodes were isolated from eight to ten-week-old mice and prepared to single cell suspension. Subsequently, cells were washed 2x in PBS/BSA/2mM EDTA and filtered through 100 µm and 70 µm cell strainers. Between washes cells were centrifuged at 1200rpm for 10 minutes. Next, cells were stained by anti-mouse CD4 FITC antibody in BS/BSA/EDTA. Following an antibody staining step, cells were washed in PBS/BSA/EDTA and resuspended in 90 µl PBS/BSA/EDTA and anti-FITC multisort beads were added in ratio 1:10, followed by incubation for 20 min in the fridge. After incubation cells were washed in PBS/BSA/EDTA and resuspended in 5 ml PBS/BSA/EDTA. Bead-labelled cell suspension was applied through a pre-separation filter to a PBS/BSA/ EDTA equilibrated LS separation column. The column was washed three times with 5ml BS/BSA/EDTA. $CD4^+$ cells were eluted from the column with 7 ml PBS/BSA/EDTA. $CD4^+$ cell suspensions with purity of more than 98% were centrifuged and resuspended in 1 ml PBS/BSA/EDTA containing 25 µl of release reagent in order to release $CD4^+$cells from the magnetic beads. Following 30 min incubation in 4^{o}C, 2 ml of PBS/BSA/EDTA was added to the release buffer with cells and applied to an equilibrated LS separation column to separate magnetic beads from $CD4^+$ cells. $CD4^+$ cells were washed out of the column with 2x 3ml of PBS/BSA/EDTA. Obtained cells were counted and resuspended to the end concentration of 100 µl PBS/BSA/EDTA per 10^7 cells. To isolate naïve cells, anti-CD62L microboeads were added in a ratio of 1:15 and incubated 20 min in the fridge. Following the incubation, cells were washed with PBS/BSA/EDTA and resuspended in 5 ml PBS/BSA/EDTA. Suspension was again applied to new equilibrated LS separation column to separate $CD62L^+$ cells. The column was washed 4 times with 3ml. Column was removed from the magnet and $CD62L^+$ cells were eluted with 7 ml PBS/BSA/EDTA. Naïve cell purity was analyzed by flow cytometry after staining with anti-mouse CD62L APC. Naïve ($CD4^+CD62L^+$) cell suspensions with a $CD4^+CD62L^+$purity of

more than 97% cells were used for T culture. In case of insufficient purity, cells were pulled through one more LS separation column.

3.2.2 T helper cell culture

CD4+ CD62L+ ("Naïve") T cells and CD4+ T helper cells were cultured at a concentration of 2,5 x 10 E6 cells/ 5 ml in a 6- well plate using RPMI supplemented with 10% fetal calve serum (FCS), 300 µg/ml glutamine, 100 U /ML penicillin, 100 µg/ml streptomycin and 50 µM β-mercaptoethanol, at 37 °C and 5% CO_2 atmosphere and 4% oxygen, if not stated otherwise. If using mice carrying wild type TCR, cells were stimulated polyclonally with anti-CD3/ anti- CD28. Prior to culture seeding, culture plate was coated with 3 µg/ml anti-CD3 antibody in PBS for 3 h at 37 °C in 5% CO_2. 1,5 µg/ml of soluble anti-CD28 antibody was added to the polarizing RPMI medium. To induce Th1-polarization, 5 ng/ml of IL-12, 10 ng/ml of IL-2 and 10 µg/ml of anti-IL4 antibody was added to the RPMI medium. To induce Th17 polarization 10 µg/ml of α-IFN-γ, 10 µg/ml of α-IL-4, 20 ng/ml of IL6, 20 ng/ml of IL-23 and 1 ng/ml of TGFβ was added to RPMI medium. After 48 h of stimulation cells were removed from the plate-bound anti-CD3 antibody. Viable Th1 and Th17 cells were harvested 5-6 days after stimulation via Ficoll density gradient centrifugation for 20 min at 2000rpm. If using TCR transgenic OTII mice ($Twist^{fl/fl}$ CD4-$Cre^{+/-}$ x OTII/$Twist^{wt/wt}$ CD4-$Cre^{+/-}$ x OTII) lymphocytes were stimulated with 1 µg/ml of cognate $OVA_{327-339}$ peptide that was added to culture polarizing medium. All cell cultural cytokine mixtures were steril-filtered before applied to cells. In all cultures, lymphocytes were co-cultured together with irradiated (30 Gy) CD90.2 depleted splenocytes as APCs from C57BL/6 mice at a ratio of 5:1. For a repeated "chronic stimulation", viable T_H cells were harvested by Ficoll density gradient centrifugation, repeatedly stimulated every 5-6 days under the original conditions 2-3 times.10 ng/ml murine IL-2 was added to the culture for second and further stimulation. Small proportions of T cells were taken for differentiation control by intracellular cytokine staining (3.2.3) and RNA extraction (3.2.4).

3.2.3 Mitogenic restimulation of T helper cells, intracellular cytokine staining and surface staining

5 x 10^5 T helper cells were restimulated by the TCR mimicking reagents PMA (10 ng/ml) and Ionomycin (1 µg/ml) for a total of 5-6 hours in growth medium. After 1 h, 5 µg/ml Brefeldin A (Bref A) was added to the cells to block exocytosis. After stimulation, cells were washed with cold PBS and stained for 15 min in the fridge in PBS containing a fixable live/dead discrimination die. Following 2x washing with PBS/BSA, cells were fixed with

Cytofix/cytoperm buffer for 20 min at 4 °C. Afterwards, cells were washed once with cold PBS/BSA/EDTA. In case of simultaneous staining of transcription factors and cytokines, cells were incubated in 0,01% Triton X-100 10 minutes on ice, washed 1x with Perm/wash buffer, than re-fixed with Cytofix/ cytoperm buffer for 5 minutes in RT and washed 1x with BD Perm/wash buffer. Cytokines/ surface markers/ transcription factors were stained with fluorescently labelled antibodies in 1X BD Wash/Perm buffer for 30 min at RT. After washing with 1X BD Wash/Perm buffer, cells were resuspended in PBS/BSA/EDTA and analysed by the MACSQuant Analyser.

3.2.4 RNA isolation

To induce Twist1 transcription, cultured cells were re-stimulated with PMA (10 ng/ml)/ Ionomycin (1 µg/ml) for 5 hours. Total RNA was isolated with RNeasy kit for transcriptome analysis, Direct-zol RNA kit for routine RT –PCR according to manufacturer's instructions and RNA concentration and quality was determined by Nanodrop -spectrometry.

3.2.5 Reverse Transcription

200 ng - 1µg of mRNA was reversely transcribed by Taqman Reverse Transcription kit according to manufacturer's recommendations. The reverse transcription reaction consists of the following steps:

- Specific annealing for 10 min at 25 °C,
- Elongation of complementary DNA (cDNA) for 40 min at 48 °C,
- Inactivation of Reverse Transcriptase for 5 min at 95 °C.

For each sample the following reaction mix was used:

	Concentration	µl / sample
Oligo dT Primer	10 mM	0.5
Random Hexamer Primer	10 mM	0.5
dNTPs	25 mM	4
Reverse Transcriptase	50 U / µl	0.5
RNAse inhibitor	20 U / µl	0.4
RT buffer	10x	2
$MgCl_2$	25 mM	4.4
RNA sample		7.7
		20 µl

3.2.6 Quantitative Real Time PCR

Quantitive RealTime-Polymerase Chain Reaction (qRT-PCR) was used to determine the specific amount of the mRNA of interest at a distinct time point by means of fluorescent signal intensity. For normalization the gene expression to the total mRNA, target gene values were compared to values of mRNA for HPRT. SybrGreen-qRT-PCR method have been used for for mRNA detection.

The qRT-PCR reaction consists of the following steps:

- 10 min at 95 °C : initial polymerase activation
- 10 sec at 95 °C :denaturation
- 10 sec at primer specific temperature (see Table 1): annealing
- 10 sec at 72 °C : elongation

For quantitation, cDNA was diluted 5x and used in the following reaction mixture:

	Concentration	µl / sample
cDNA		1
Forward primer	5 µM	0.25
Reverse primer	5 µM	0.25
SybrGreen reaction mix	n. a.	0.5
$MgCl_2$*	25 mM	Depending on primer pair
RNAse-free Water		Depending on primer pair
		10 µl

* $MgCl_2$-concentrations have been determined during primer establishment (see Table 1)

Table 1 : Primer sequences of target gens, annealing temperature and MgCl2 concentration

Gene - primer pair	Forward primer (fw)	Reverse primer (rv)	Annealing	$MgCl_2$
HPRT (mouse)	tcctcctcagaccgctttt	cataacctggttcatcatcgc	65°C	3 mM
HPRT (human)	acccttttccaaatcctcagc	gttatggcgacccgcag	60°C	3mM
Twist1 (mouse)	cgcacgcagtcgctgaacg	gacgcggacatggaccagg	65°C	2 mM
Twist1 (human)	ggcacccagtcgctgaacg	gacgcggacatggaccagg	65°C	2 mM
Bim (mouse)	cccggagatacggattgca	aacaccctccttgtgtaagtttcgt	60°C	2 mM
IL10 (mouse)	aggcgctgtcatcgatttct	atggccttgtagacaccttgg	60°C	4mM
PRDM1(mouse)	gacgggggtacttctgttca	ggcattcttgggaactctct	60°C	3mM

Trp53inp1(mouse)	actcacgggcacagaaatgg	gggcgaaaactcttgggttg	60°C	3mM
Bnip3(mouse)	gaagcgcacagctactctca	tccaatgtagatccccaagcc	60°C	4mM
P2X7R(mouse)	aggccaagaagttccaaccta	tccattgagagcatggcttctt	60°C	4mM
Bcl6(mouse)	ctgtgaaatctgtggcactcgc	cagcggccgacacgcggtattgc	60°C	3mM
PD-1(mouse)	cgtccctcagtcaagaggag	gtccctagaagtgcccaaca	60°C	4mM
Glut3(mouse)	tgtaggacccgaggaacact	gatggggtcaccttcgttgt	60°C	4mM
Glut1(mouse)	Gtgacgatctgagctacggg	tcaccttcttgctgctggg	60°C	4mM
Pdk1(mouse)	tgcgacaagagttgcctgtt	ggctttggatataccaactttgcac	60°C	2mM
ICOS(mouse)	aaacaacccagacagctccc	gcagcagagctgggattcata	60°C	4mM
Myc(mouse)	ttgaaggctggatttcctttgggc	tcgtcgcagatgaaatagggctgt	60°C	3mM
PRDM1(mouse)	gacgggggtacttctgttca	gcattcttgggaactgtgt	60°C	3mM
Gpx4(mouse)	acctggacgccaaagtccta	ggctgagaattcgtgcatgg	60°C	3mM

Primers were synthesized by and purchased from TIB Molbiol Berlin.

3.2.7 Glucose uptake assay

Viable T helper cells were harvested by Ficoll density gradient centrifugation and washed in PBS/BSA. Subsequently, cells were seeded into a 96-well plate coated previously with 3 µg/ml of anti-CD3 in concentration 3x10 E5 cells per well and stimulated with for a 5 h in a RPMI complete Th1 polarizing medium with soluble 1,5 µg/ml of anti- CD28, as described in the section 3.2.2. After the incubation, cells were centrifuged down, medium was aspirated and 100uL of glucose-free RPMI Th1 media with 1,5 µg/ml of soluble anti-CD28 antibody was added to the cells and incubated for 60 minutess. After incubation, solution of 100 uL of glucose-free RPMI with 300uM of 2-((7-nitro-2,1,3-benzoxadiazol-4-yl)amino)-2 deoxyglucose (2-NBDG) was added to the well to reach concentration of 150 µM. Cytochalasin B has been shown to inhibit GLUT1, 2, 3 and 4 and therefore block transport of glucose to the cell. Therefore, well with no 2-NBDG and well with 150uM of 2-NBDG+ 30 µM Cytochalasin B were used as controls. Cells were incubated for 30 minutes at 37^{o}C. After incubation, cells were washed 2x in the PBS with centrifugation 1200 rpm, resuspended in PBS and maintained at 4 ^{o}C. Data were acquired by measurement of fluorescence in FL-1/FITC channel immediately using a flow cytometry after exclusion of dead cells by PI staining.

3.2.8 Seahorse Assay

Oxidative phosphorylation and glycolysis can be estimated relatively easily and quickly from the rates of linked reactions: extracellular acidification resulting from glycolytic conversion of uncharged glucose to 2 lactate− plus 2 H+ and oxygen consumption to oxidize substrates such as glucose and fatty acids to support oxidative phosphorylation. Seahorse XP analyser can measure both of output reactions. Viable T helper cells were harvested by Ficoll density gradient centrifugation and washed in PBS/BSA. Subsequently, cells were washed 3x with glucose free RPMI assay media, pH 7,35, and seeded in the density of 2 x10 E 5 cells per well in 180 µl glucose free RPMI, culture medium (pH 7,35) onto an XF96 Seahorse culture microplate which was coated a day in advance for 2 h in RT with 13 µL of 100µg/ml Poly-D-Lysine solution per well and washed 2x with 500 µL of ddH_2O. No cells were plated in the four edge rows. Prior to the assay, cells were put for 90 minutes into 37°C incubator without CO_2. In the meantime assay compounds were prepared as indicated in Table 2. Chemicals were diluted in Seahorse assay medium and were loaded to the ports of the equilibrated XF96 four-port sensor cartridge. Acidification rate (ECAR) and consumption rate was monitored over time for 180 min with injection of glucose after 15min, and injection of PMA/Ionomycin at 35 minute. Assay was performed in 21% oxygen.

Table 2 Compounds used for Seahorse assay:

Compund	Port	Stock conc	Conc. In port	Volume in port	Conc. On cells
Glucose	A	1M	100 mM	20 µL	10mM
PMA/Iono	B	1mg/ml	10 µg/ml Iono 100ng/ml PMA	22 µL	10 ng/ml PMA and 1 µg/ml Ionomycin

3.2.9 Metabolic survival assay

3.2.9.1 Mouse T cell culture

Viable CD4 T helper cells were harvested by Ficoll density gradient centrifugation and washed in PBS/BSA. 96-well plate was coated with 3 µg/ml anti-CD3 for 3 hours at 37°C. Cells were plated in complete RPMI medium with 1,5 µg/ml soluble anti-CD28 and

polarization cytokines plus inhibitors listed in Table 3. Cell number was monitored over time in 2 day interval using exclusion of dead cells with 100 ng/ml of Propidium Iodide or 100 ng/ml of DAPI dye. Detection was done by flow cytometer.

3.2.9.2 Human T cell culture.

Synovial fluids were taken by puncture of joints of patients suffering from juvenile idiopathic arthritis. At first, synovial fluid was centrifuged with rotational speed 1500 rpm. Cells were resuspended in PBS/EDTA and depleted of $CD14^+$ cells using 2x MACS column and anti-CD14 magnetically labelled antibody. Sorted $CD4^+$ T cells were plated in RPMI medium containing a human serum, antibiotics and inhibitors to monitor cell number over time using CD4 antibody and DAPI. Proliferation was monitored by plating equal number of cells labelled with Cell Proliferation Dye eFluor™ 670 (eBioscience). To detect cytokines, $CD4^+$ $CD14^-$ cells were re-stimulated by PMA/Ionomycin for 5,5 hours, BreA was added 1 hour after initial stimulation. Cells were fixed according to protocol described in 3.2.3. Paired blood was taken from diseased donors as a control and processed identically, except the cells were first isolated by density gradient separation using Ficoll. Synovial and control cells were than FACS sorted by surface expression of $CD3^+$ $CD4^+$ $CD14^-$ $CD45RO^+$ PD1+ and $CD3^+$ $CD4^+$ $CD14^-$ $CD45RO^+$ PD1- cells to two groups. Cells from two cell groups were plated in identical cell number (1,5 x10E5 per 96 well- plate) in a complete control medium or complete medium containing 150μM Etomoxir. Cell number was counted over time using MacsQuant. Dead cells were excluded using DAPI. To isolate RNA, sorted cells were washed in PBS and lysed in Zymo lysis buffer for RNA extraction described in section 3.2.4.

Table 3- Chemical inhibitors of metabolic pathways

Inhibitor	Final concentration	Inhibited catabolic pathway
DON	50μM	glutaminolysis
2-deoxyglucose	2 mM	glycolysis
oligomycin	2 μM	OXPHOS
Etomoxir	150 μM	Fatty acid oxidation

3.2.10 Global transcription analysis of Twist1 targets by microarray assay

Transgenic Twist$^{loxP/loxP}$ x OTII and Twist1 $^{wt/wt}$ x OTII murine naïve T cells were cultured under Th1 polarizing conditions for 4 rounds of stimulation (**Figure 3-1**). TAT – Cre was added on day 21 of the culture. TAT - Cre was purified in advanced by Large Scale Purification Under Native Conditions using protocol described by (Peitz et al. 2002). TAT-CRE is a recombinant cell-permeant fusion Cre - recombinase protein consisting of TAT sequence and a nuclear localization sequence (NLS). It catalyzes the site specific recombination event between two loxP DNA sites. Cells were isolated and cultured as described in paragraph 3.2.1 and 3.2.2. Culture was performed under 21% oxygen levels.

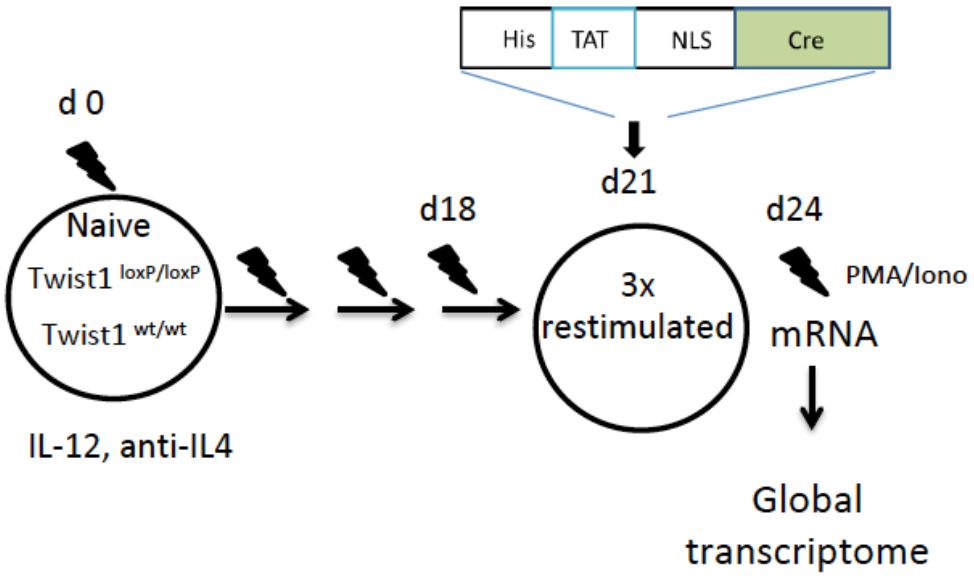

Figure 3-1 Schematic overview of the steps involved in gene expression profiling of Twist1 deficient repeatedly stimulated T cells:

CD4+ CD62L^{hi} cells were stimulated *in vitro* for 4 rounds of stimulation (lasting 6 d each) under T_H1-polarizing conditions, with splenic APCs and $ova_{327-339}$, On day 21(day 3 after re-stimulation), synthetic CRE with attached nuclear localization signal was added to the culture. On day 24, life cells were collected and re-stimulated for 3,5h with PMA/Ionomycin. RNA was isolated using RNeasy Mini Kit (Quiagen).

Purified RNA of Twist1 Th1 deficient cells (Twist1 $^{loxP/loxP}$ x OTII) and control Twist1 $^{wt/wt}$ x OTII cells was hybridized to Affymetrix GeneChip® Mouse Genome 430 2.0 arrays according to the manufacturer's instructions by Heidi Schliemann. The 10 µg of RNA of the cells of interest is isolated, reverse-transcribed to cRNA, labeled and incubated with the array. Arrays contain spots of short oligonucleotides representative of the coding regions of all genes. Complementary sequences hybridize and the amount of hybridized RNA is later quantified by fluorescence detection. Dr. Joachim R. Grün analyzed differentially regulated genes.

3.2.11 Lipid peroxidation

BODIPY 581/591 C11 (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid) was added to T cells treated with metabolic inhibotors in 96well plate at a final concentration of 3 µM inhibitor in RPMI, serum/β-Me/FREE medium and allowed to incubate for 40 min at 37^0C. The cells were washed in PBS/BSA. Dead cells were excluded by DAPI staining and live cells were assessed using an MACS Quant flow cytometer. Excitation was achieved with an argon laser (488 nm). Green fluorescence was detected with an FL1 band pass (BP) filter 530/30.Data were collected from a minimum of 50 000 cells.

3.2.12 Lipid content

To measure lipid content 250,000 cells were plated onto a 96-well plate in quintuplicates resuspended in conditioned serum/β-Me containing medium, 37°C. At the time point of the measurement 50µl aliquot was removed for staining with 400nM Bodipy 505/515 into serum/β-Me/FREE conditioned medium and incubated 40´ 37°C. Next, cells were washed and resuspended in cold PBS/BSA and measured DAPI negative cells for FL1 fluorescence.

3.3 Statistics

All statistical analyses used the Mann-Whitney-Test for unpaired data, unless stated otherwise. A *P* value of equal or less than 0.05 was considered significant.

4 Discussion

Rheumatic diseases significantly affect patient´s life. Throughout progression of an autoimmune synovial arthritis, formation of ectopic lymphoid structures supports amplification mechanisms that serve to sustain the lifelong inflammatory process (Humby et al. 2009). Within those structures, CD4+ T cells are particularly harmful as they are potent drivers of autoimmune response (Rao et al. 2017a), resisting therapeutic intervention (Eggleton et al. 2010; Scott et al. 2010). Resistance to cell death likely results from collection of factors, such as interaction with synovial stromal cells (Salmon et al. 1997) or downregulation of pro-apoptotic factor Bim (Haftmann et al. 2015). Nevertheless, metabolic adaptation as a mean to survive is merely studied. As a repeated stimulation is associated with acquisition of properties towards pathogenic phenotype (Lee and Lee 2016), we reasoned that differences in the duration of antigen stimulation could alter the metabolic phenotype. Changes introduced by repeated stimulation involve CD28 downregulation or upregulation of PD and CTLA4 surface molecules (Walker 2017). These markers are associated with metabolic switches (Patsoukis et al. 2015; Frauwirth et al. 2002). For the first time, we explore metabolic properties of *in vitro* repeatedly stimulated Th1 cells. Observed phenotype in this model was subsequently translated to metabolism of CD45RO+ CD4+T cells isolated *ex vivo* from auto-inflamed synovial fluid of Juvenile Idiopathic Arthritic patients. RNA analysis revealed that the pathogenic subset (CD45 RO + CD4+ CXCR5- PD1+) of synovial CD4+ T cells express higher levels of transcription factor *Twist1,* hallmark of pathogenic Th1 cells.

Cytokine secretion from CD4 T cells in synovial environment appears to be low (Firestein and Zvaifler 2002). Furthermore, Twist1 is elevated in biopsies of patients that are both treated and un-treated with immunosuppressive therapy. This suggests that cytokine inhibition, previously described function of Twist1 in Th1 cells, might not be the main one in the inflammatory milieu. Twist1 functions as a regulator of energy homeostasis and fatty acid metabolism in adipocytes (Pan et al. 2009; Pettersson et al. 2010) and therefore we explored role of Twist1 in metabolism strategy of CD4 T cells that survive and persist in the inflamed tissue.

4.1 Chronically activated Th1 cells adapt to more efficient metabolic pathway

Throughout development, T cells present high flexibility in navigation of their metabolic programs according to their immediate needs. This ability to adapt is retained during their life cycle (Blagih et al. 2015; Weis et al. 2017). Although immune cells spend significant amount of time in the blood, where nutrients are relatively abundant, inflamed synovial environment of rheumatoid joint lacks blood supply due to a defective vascularization and oedema, leading to a low levels of oxygen and nutrients (Maciolek et al. 2014). Energy demands for inflammatory CD4+ T cells are energetically challenging and therefore cells need to adjust their metabolism to survive. To address metabolic adaptations to chronic stimulation in a nutrient depleted environment, we first employed an *in vitro* model of chronically stimulated Th1 cells using murine CD4 T lymphocytes (Figure 2-1). Murine Th1 cells were co-cultured for several rounds of stimulation together with an antigen presenting cells. In order to imitate the *in vivo* conditions as closely as possible, we cultured cells in 4% oxygen. Upon repeated stimulation, Th1 cells decrease glucose uptake (Figure 2-2) and decrease their glycolytic levels (Figure 2-3). However, cells still retain residual glucose uptake in comparison to blocked control (Figure 2-2). Residual carbon intake is not only used for cytokine production and homeostatic proliferation (Tripmacher et al. 2008), but importantly, for synthesis of triacylglycerides (TAG). TAG are necessary for memory survival (Cui et al. 2015). Decrease of glycolytic metabolism connected to a repeated stimulation of CD4 T cells has been observed previously in graft versus host disease (GVHD), where lower glucose uptake was associated with decrease of CD28 expression (van Baarle et al. 2005). Cells metabolism shifted towards fatty acid oxidation (Byersdorfer et al. 2013). Therefore, we tested how glycolytic and oxidative phosphorylation rates change with repeated stimulation in Th1 cells.

CD4 T cells in memory or chronic (auto-) inflammatory diseases must often endure long period of quiescence and nutrient (and glucose) depleted environment. How Th1 cells respond once glucose is not available? If Th1 cells lost access to extracellular sources of glucose, three times stimulated Th1 cells had higher oxidation levels than their once stimulated Th1 counterparts (Figure 2-3b). This is an indicatiton, that CD4 memory T cells employ similar mechanism as CD8 T cells. CD8 memory T cells store intracellular lipid reserves that are oxidized as a energy source for survival (Michalek et al. 2011).

However, it should be noted, that glycolytic rates of cells that lost an access to extracellular sources of glucose were slightly, but significantly higher also in resting repeatedly stimulated cells. It remains to be investigated what could be a source of such acidification. CO_2 generated in active TCA is a source of hydrogen protons which might cause the acidification of a medium. However, no data are available at the moment and the exact cause remains to be found.

If glucose is available to cells, glycolysis was lower in repeatedly stimulated Th1 cells than in once stimulated than (Figure 2-3c), indicating that repeated stimulation induces a switch in metabolism. Optimisation of metabolism towards actual energy needs is logical: while glycolysis secures 2 molecules of ATP per oxidized glucose, per one molecule of palmitic acid oxidation secures 129 molecules of ATP. The oxygen consumption was higher repeatedly stimulated T cells than once stimulated cells, indicating increased capacity of repeatedly stimulated cells to oxidize its substrates after repeated stimulation in both resting and activated phase (Figure 2-3).

Previously effector T cells were found to be dependent in survival, cytokine secretion and cytokine production on glucose utilization (Michalek et al. 2011; Yaqoob and Calder 1997; Chang et al. 2013). Observed lower levels of glucose uptake and lower glycolytic levels in repeatedly stimulated cells led us to investigate how survival is affected if glucose is limited.

We hypothesized that decreased level of glycolysis and glucose uptake mean metabolic shift towards fatty acid oxidation. To this end, we measured survival in glycolysis and fatty acid oxidation - limiting conditions (Figure 2-4). Repeatedly stimulated Th1 cells were significantly less dependent in survival on glucose than were their once stimulated counterparts (Figure 2-4a). Accordingly, a long term of *in vitro* survival of repeatedly stimulated CD4 Th1 cells is completely abolished if cells are treated with fatty acid oxidation inhibitor Etomoxir (Figure 2-4b). Interestingly, repeatedly stimulated cells treated with Etomoxir had higher survival on day 2 and day 3. This is possibly due to the cell metabolic flexibility - higher glucose utilization is forced by inhibition of fatty acid oxidation (Figure 2-4c). PD-1 mediates oxidation of fatty acids (Patsoukis et al. 2015) and is upregulated in GVHD cells, that are dependent on fatty acid oxidation. Accordingly, we measured *pd-1* mRNA expression (Figure 2-5) in repeatedly activated Th1 cells and in contrast to once activated Th1 cells, *pd-1* mRNA expression was increased. Altogether, results in section 2.1 confirm ability of CD4 + T lymphocytes to adapt to chronic stimulation *in vitro*. In this experimental set-up, phenotypic characteristics that were acquired throughout a long term *in vitro* culture did not allow a long term utilization of glycolysis as cells treated with FAO inhibitor Etomoxir did not survive after day 3 (Figure 2-4b) indicating high dependency of repeatedly simulated Th1 cells on FAO.

4.2 *ex vivo* JIA autoimmune CD14- CD4+ T cells secrete IFN-γ and prefer oxidative metabolism for survival

In order to translate murine *in vitro* studies to an autoimmune rheumatoid disease, we took synovial punctate from Juvenile Idiopathic Arthritis (JIA) patients. JIA is the most common form of persistent arthritis in children. Even if the cause of disease are not fully disclosed and there exist several subtypes (see section 1.3 for detailed description) chronic adaptive immune responses are involved in the pathogenesis, as indicated by the presence of T and B lymphocytes infiltrating the synovial membrane of inflamed joints (Murray et al. 1996) and share many similarities with rheumatic arthritis. T-cell infiltrates, in both rheumatic arthritis and juvenile idiopathic arthritis, predominantly consist of CD4+Th1 cells which have been thought to have a central role in the pathogenesis of the disease (de Jager et al. 2007) while Th17 subset presence is still debated (Miossec 2011). Th1 phenotype present in the inflamed tissue of RA patients is also supported by the presence of IFN-γ-secreting $CD4^+$ T cells in the synovium of patients with RA (Nanki and Lipsky 2000). In order to investigate the phenotype of CD4+ T cells, we isolated out of the synovial JIA punctate CD14 - CD4+T cells and stained them for surface expression of effector and memory markers CD45 RA and CD45 RO. Than, we re-stimulated CD14- CD4+T cell with PMA/ Ionomycin in 4% oxygen, as hypoxic oxygen tension has been measured in synovial joint (Gaber et al. 2009),

Flow cytometry showed that majority of T cells have memory (CD 45 RO+ CD45 RA-) phenotype, in contrast to the T cells obtained from patients' blood (Figure 2-6), reflecting past or present TCR stimulation. In accordance with previous studies (de Jager et al. 2007), after PMA/ Ionomycin restimulation, CD4 T cells expressed IFN-γ and TNF- α and minimum of IL17A, reflecting prevalence of Th1 associated cytokine imprinted genes. Of note, other IL17 family members, particularly IL17F have not been measured, so possibility of a IL17 isoform is not excluded. In average 9% of IFN-γ expressing cells also co-expressed IL10 (**Figure 2-7**b), induced as a compensatory mechanism of Th1 cells in chronic immune response to alloantigen's (Anderson et al. 2007; Roncarolo et al. 2006) to induce peripheral tolerance. Supportive of Th-1 phenotype, CD14- CD4+ CD45 RO+ T cells isolated from synovial fluid had higher T- bet expression than CD14- CD4+ CD45 RO+ or CD14- CD4+ CD45 RO- T cells isolated from patients' blood (**Figure 2-7**c).

To address metabolic properties of these cells, we were interested in how isolated cells response to metabolically challenging environment. We measured increased PD-1 expression in CD45 RO+ CD4+ CD14- T *ex vivo* isolated cells from JIA patients in contrast to the patients´ blood (Figure 2-8). Interestingly, stimulation with PMA/Ionomycin did not

influence expression of PD1, suggesting that cells might be upon isolation in activated state. As CD4 T cells expressing PD-1 have been shown to shift preferred metabolism towards fatty acid oxidation in *in vivo* models of GVDH and in murine *in vitro* model of ubiquitous antigen exposure(Byersdorfer et al. 2013), we employed metabolic inhibitors of glycolysis, glutaminolysis and fatty acid synthesis to determine survival sensitivity to metabolic inhibition. In contrast to a CD4 T cells isolated from blood, CD14- CD4+ T cells isolated from synovial fluid of JIA patients survive a long period of time in *in vitro* culture (Figure 2-9), suggestive of their robust survival potential. Even though cells in *ex vivo* culture proliferated very little (Figure 2-10), stimulation with CD3/CD28 was critical for survival after day 4 (Figure 2-9b) of the *in vitro* culture. Two days after activation anti-CD3/anti-CD28 stimulated and un-stimulated synovial CD4+ CD14- T cells treated with glycolysis inhibitor 2DG had on average 20% lower survival than control cells (Figure 2-9b), pointing towards glycolysation metabolism in the activation phase if glucose is available. Nevertheless in a long term survival, synovial CD4 T cells were most sensitive to inhibition of fatty acid synthesis and oxidative phosphorylation (Figure 2-9b, Day 8, anti-CD3/anti-CD28). Collectively, these data indicate that synovial CD4+ T cells do employ different metabolic pathways in different stages of activation to supply energy and biomolecules for survival, homeostatic proliferation and cell renewal.

4.3 PD1+ CXCR5- can be selectively eliminated by fatty acid oxidation inhibitor Etomoxir *in vitro*

Peripherally expanded cell population of CD4+ CD45RO + CXCR5- PD1+ T cells have been shown to drive inflammation by providing B cell instruction via stimulatory signals such as IL-21, CXCL13, ICOS and MAF (Rao et al. 2017a). Due to its pathogenicity, these cells are unique targets to reduce inflammation. Any potential therapeutic interventions to relieve the inflammation require cell specificity or at least a higher degree of sensitivity. To study sensitivity of synovial CD4+ CD45RO + CXCR5- PD1+ T cells to metabolic inhibition, we sorted out and cultured CD4+ CXCR5- PD1+ from CD4 + CXCR5- PD1- cell populations in the presence or absence of fatty acid oxidation inhibitor Etomoxir (Figure 2-11). CD4 + PD1+ CXCR5- T cells cultured in a control medium had 60% survival rate in comparison to 20% survival rate of CD4 + CXCR5- PD1- cells, pointing to the survival advantage of PD1+ T cells. This might indicate, that PD-1, which is overexpressed in synovial cells from patients with rheumatic arthritis, might protect autoreactive T cells from undergoing apoptosis (Lin et al. 2004; Wan et al. 2006). To show this, we added Etomoxir to the culture. We were able to eliminate survival advantage of PD1+ T cells. Cells were unable to survive till day 4, in comparison to CD4+ CXCR5- PD1- cells, that had comparable survival rates on day 4 in

Etomoxir containing medium as in control culture (**Figure 2-11**). Evidence of selective inhibition of pathogenic CD4+ CXCR5- PD1+ expanded population with Etomoxir is a promising initial experiment that will be expanded to both *in vitro* PD-1 blocking or stimulation with PD-1 ligand with visualization of active intracellular signalling molecules and transcription factors.

4.4 CD4 + CD45 RO+ PD1+ CXCR5- cells express Twist1 that mediates inhibition of metabolic activation

We have previously shown that expression of transcription factor Twist1 is increased in cells isolated from inflamed rheumatoid joints (Niesner et al. 2008). Twist1 has been shown to induce microRNA148a, which regulates expression of the proapoptotic protein Bim (Haftmann et al. 2015) and therefore supporting cell persistence of repeatedly “pathogenic” CD4 T cells. By means of transcriptional analysis we show that CD4+ CD45 RO+ PD1+ CXCR5- cells isolated from JIA patients expresses higher mRNA levels of Twist1 than their CD4+ CD45 RO+ PD1- CXCR5- counterparts (Figure 2-12). What is a role of Twist1 in pathogenic synovial CD4 T cells? It has been shown that T cells receiving PD-1 signals decrease glycolysis and enhance FAO, which dampens their effector function while promoting longevity. Does Twist1 contributes to this phenotype? Twist1 was shown to be involved in a regulation of metabolism in diverse tissue cell types, such as brown adipose tissue cells, white tissue adipose cells or skeletal muscle cells. In brown adipocytes Twist1 acts as a negative feedback regulator of PPAR-δ /PGC1α signalling, major regulators of energy, glucose and fat metabolism (Maruyama et al. 2016). Twist1 inhibits dissipation of energy produced by oxidation in form of heat (Pan et al. 2009). In white adipose tissue Twist1 positively regulates fatty acid oxidation and anti-inflammatory function (Dobrian 2012; Pettersson et al. 2011). In muscle cells, Twist1 regulates glycogen storage (Mudry et al. 2015b). To address Twist1 metabolic function in repeatedly stimulated Th1 cells, we used CD4 conditional Twist1 deficient mice. Since Twist1 protein expression in lymphocytes peaks 5 hours after activation (Niesner et al. 2008), we aimed our focus on metabolism of repeatedly stimulated cells in this time frame when cell increase its glycolytic rates. To this end, we measured acidification rates (surrogate marker of glycolysis) and oxidative consumption (surrogate marker of oxidative phosphorylation) using Seahorse XP analyser in once and repeatedly stimulated /control Twist1 wild type and Twist1- deficient Th1 cells. Results indicate that Twist1 downregulates glycolysis in both resting Th1 and resting Th17 cells. After activation with PMA/ Ionomycin downregulation is specific to once and repeatedly activated Th1 cells (Figure 2-13a,b), not Th17 (Figure 2-13c). Oxidative phosphorylation was

also downregulated by Twist1 in both once and repeatedly stimulated Th1 cells (Figure 2-13d,e), with higher downregulation of oxidative consumption in three time stimulated Th1 cells. This effect was not observed in Th17 cells (Figure 2-13f). Even though the glycolysis is a major pathway, OXPHOS is still active - significant portion of glycolytic derived pyruvate is oxidized in mitochondria during T cell activation. Therefore, it is plausible that higher levels of oxidation in Twist1 deficient cells are caused by increased level of glycolytic derived pyruvate entrance to TCA cycle. Indeed, if cells are forced to increase glycolytic rates due to an inhibition of fatty acid oxidation by Etomoxir, we observed enhanced difference in glycolysis between WT and Twist1 deficient cells (Figure 2-20b). In summary, Twist1 appears to inhibit metabolic activation, which supports its inhibitory properties on cytokine expression upon activation (Niesner et al. 2008), because metabolism is tightly interconnected with cytokine expression. Glycolytic intermediate GAPDH, if not engaged in glycolysis, binds to an IFN-γ promoter and inhibits IFN-γ expression (Chang et al. 2013).

4.5 Transcriptional profile of Twist1 deficient cells suggest role in activation anabolism and protection against oxidative species in repeatedly stimulated Th1 cells

In order to get further insight into Twist1-mediated transriptional regulation of three time stimulated Th1 cells, we cultured transgenic OTII Twist1$^{wt/wt}$ anf Twist1$^{fl/fl}$ for 4 rounds of stimulation. 3 days before last re-stimulation, recombinant Tat-Cre protein was applied to the culture (Figure 3-1). This strategy omits secondary effects of a long-term culture of Twist1 deficient cells. We observed relatively low number (73) of differential regulated genes. We observed no Th1 specific cytokine genes regulated by Twist1. This is in contrast to once stimulated cells, where Twist1 regulates gene expression of several Th1- associated cytokines such as *IFN-γ, TNF-α* and *IL-2* in Th1 cells (Niesner et al. 2008; Pham et al. 2012). This finding was confirmed by flow cytometry measuring cytokine expression in three times stimulated Twist1 deficient and WT confirmed this finding (Figure 2-14). These unexpected results likely originate from repeated stimulation that require overall change in global transcriptome and therefore also change availability of protein binding co-factors, which are determining Twist1 function (Qin et al. 2012). Limitation of artificial *in vitro* culture environment also needs to remain considered, as up to 98% of repeatedly stimulated T cells secrete IFN-γ, which is not observed in cells isolated *ex vivo* and/or restimulated polyclonally with anti-CD3/anti-CD28. Twist1 has been shown to be functional also in effector Th17 cells and T_{FH} cells, where its expression is induced by STAT3. However, Twist1 expression is not upregulated by repeated stimulation of Th17 or Th2 cells, in contrast to Th1 cells, suggesting

that alternative function might develop with repeated stimulations. Therefore, Twist1-mediated suppression of cytokines might not be as relevant in repeatedly stimulated Th1 cells as in effector T cells. Interestingly, we observe several differentially expressed genes that are also regulated by master transcription factor Bcl6 such as *Icos, Tnfsf9* (coding for 41BBL), *IL21* are downregulated by Twist1. This is interesting, because PD-1 expressing follicular T helper cells, where Twist1 is induced by STAT3, Twist1 acts as limiting factor in the development of cell-mediated and humoral immunity (Pham et al. 2013). Concurrently, pathogenicity of peripherally expanded cell population CD4+ CD45RO + CXCR5- PD1+ T cells have been shown to drive inflammation by providing B cell instruction via stimulatory signals such as IL-21, CXCL13, ICOS and MAF (Rao et al. 2017a). Therefore it is possible, that “new” protective function of Twist1 in repeatedly stimulated Th1 cells is to inhibit this presentation. Protective suppression of B cell instruction in synovial CD45RO+ CD4+PD1+ CXCR5- synovial T cells Twist1 might be another contributing factor to the inflammatory phenotype observed in animal model of arthritis treated with small hairpin RNA against Twist1 (Niesner et al. 2008).

Another two sets of differentially regulated genes were genes with reported function in apoptosis and metabolism. We observed upregulation of *Bnip3*. Its anti-apoptotic function stems from its metabolic regulation of mitochondria autophagy after activation induced release of BH3 factors, such as Bim, that mediate caspase induced cell death (Kroemer and Reed 2000). Cells expressing Bnip3 elude cell death by upregulating mitochondrial autophagy coupled with ROS scavenging. Lysosomal degradation of mitochondria further promotes cell survival (O'Sullivan et al. 2015; Moriyama et al. 2017). Further, Bnip3 has been shown to promote oxidation of fatty acids and maintaining mitochondrial integrity (Glick et al. 2012). Second highest differential regulated gene was *Ppp1r3b*. *Ppp1r3b* has been described so far only in liver cell, and has been shown to be upregulated in a lymphoid tissue of autoimmune mice (Zhu et al. 1993). *Ppp1r3b* codes for GSK3 targeting catalytic unit of protein phosphatase 1 (PP1). PP1 activates glycogen synthase kinase (GSK3) by dephosphorylation. PP1. It plays a major role in regulation of number of enzymes involved in glycogen synthesis, glucose and triglyceride metabolism (Ceulemans and Bollen 2004; Zhang et al. 2014). Moreover GSK3 has been shown to inhibit T-bet function and upregulate PD1 expression in CD8 T cells (Taylor et al. 2016), introducing possibility that Twist1 promotes PD1 phenotype by stabilizing GSK3 via *Ppp1r3b* upregulation. Also, interactions between Twist1 and its functional co-factors are controlled by GSK3 mediated phosphorylation during tumor progression (Lander et al. 2013). Thus Twist1 mediated regulation of *Ppp1r3b* might be mechanism to support dimerization of Twist1 with its functional partners. Twist1 also upregulates *Activating transcription factor 5* (*ATF5*) that promotes mitochondrial function and recovery from mitochondrial stress (Fiorese et al. 2016).

Glut3 and *Glut1* code for proteins mediating a glucose uptake in T cells (Gerriets et al. 2015) and *Oxct1, PAISC* and *Gpt2* genes code for proteins involved in synthesis of biomolecules. Oxt1 catalyse the formation of Acetoacetyl-CoA, which is a critical metabolite for formation of cholesterol, lipid and short-chain acyl-CoAs (MacDonald et al. 2009). PAICS (ADE2) is a synthase involved in production of purines in a pentose phosphate pathway, involved in proliferation and metastasis of prostate cancer (Chakravarthi et al. 2017). Gpt2 is a transaminase located in mitochondria catalysing the generation of pyruvate and glutamate, therefore being essential in TCA anaplerosis, crucial feature for synthesis of fatty acids (Ouyang et al. 2016). Number of differentially regulated genes by Twist1 is involved in scavenging intracellular reactive oxygen. *Gpx4* codes for a NAPDH dependent peroxidase, catalyzing oxidation of reduced glutathione by lipid peroxide, major intracellular lipid peroxide scavenger. *Rdh11* codes for a NADPH-dependent retinal reductase, playing a role in regulation of oxidative stress, exhibits an oxidoreductive catalytic activity and is involved in the metabolism of lipid peroxide derived - short-chain aldehydes (Kasus-Jacobi et al. 2003). Last, we observed single downregulation of master transcription factor of glycolysis *Myc* (Wang et al. 2011), which might be molecular background of Twist1 mediated downregulation of glycolysis observed in Seahorse studies. Moreover, co-upregulation of genes coding for proteins involved in glucose uptake with genes involved in synthesis of biomass (purines, fatty acids and triglycerides) suggest involvement of Twist1 in activation induced anaplerosis, that is essential for survival of memory T cells (Cui et al. 2015). Therefore, Twist1 might contribute to metabolic mechanism supporting memory – futility. Futility is simultaneous process of intracellularly synthesis of fatty acids and its oxidation (Weinberg and Chandel 2014), utilizing glucose and amino acids from extracellular environment (Weinberg and Chandel 2014). While futility is well studied in adipose and muscle tissue (Dulloo et al. 2004), and newly discovered in memory CD8 T cells (Weinberg and Chandel 2014) mechanism that overpass mutually inhibiting FAO vs. FAS model described in effector T cells needs to be explored. The answer should require studying localization and levels of Malonyl-CoA in different subpopulations and states, and determining the thresholds inhibiting or allowing function of CPT1.

In summary, based on herewith described transcriptional analysis Twist1 supports synthesis of lipids during activation, which might be mechanism related to the survival adaptation of a cell to its nutrient limiting environment. It is also possible that Twist1 plays an alternative protective function – inhibition of B cell instruction, which is pathogenic within synovial environment.

4.6 Role of Twist1 in fatty acid metabolism

Affymetrix analysis suggested Twist1 role in build-up of fatty acid storage during activation and protection against ROS (Figure 2-15). 24 hours after activation, we observed slightly higher lipid content in Twist1 wild-type cells, nevertheless the difference with Twist1-deficient cells was not significant (Figure 2-16a). However, later time points after activation need to be measured. In order to test functionally possible lack of fatty acids in a long term survival, we inhibited glycolysis and glutaminolysis as a two major energy producing alternative pathways to fatty acid oxidation. We hypothesized that the cell will be forced to utilize maximum of fatty acids to suffice its energy demands. As showed in (Figure 2-16b), in contrast to three time stimulated WT cells, three times stimulated Twist1 deficient cells are not capable to survive on fatty acid oxidation. Taken together, even if the molecular mechanism remains elusive, Twist1 is essential for repeatedly stimulated Th1 cell to survive on fatty acids, possible because of its instruction to build-up of lipid storage during activation, that is used to supply energy for a long term homeostatic survival.

Lipid peroxidation has been previously indicated as a potent factor in pathogenesis of rheumatoid arthritis (Seven et al. 2008), where it might mediates antigen presentation, cross presentation and release (Dingjan et al. 2016). Therefore, we aimed our investigation towards protection of the cell against oxidative species that are formed during oxidative metabolism. It was published previously that Etomoxir treatment induces lowering levels of NAPDH and subsequent increase of ROS (Pike et al. 2011; Estan et al. 2014), in spite to the fact that main OXPHOS source, fatty acid oxidation, is inhibited. We confirmed Etomoxir mediated increase of ROS in Figure 2-17. Etomoxir treatment induced high levels of superoxides and lipid peroxides 24h after activation. In a contraction phase (192 hour after activation) high levels of lipid peroxides were observed specifically in three times stimulated T cells. Twist1 deficient cells were observed to have higher levels of lipid peroxides than their WT counterparts (Figure 2-18). We hypothesized that because of downregulation of *Gpx4* mRNA in Twist1 deficient cells, Twist1 deficient cells would be sensitive to lipid peroxidation. We asked if Etomoxir treatment, causing lipid peroxidation, will affect survival of once and repeatedly stimulated Twist1 deficient cells? We normalized their survival to the control wt cell. Once stimulated Twist1 deficient Th1 cells treated with Etomoxir were hampered in survival 3 days after activation in contrast to WT cells, nevertheless cells were capable of recovery: 7 days after activation, Twist1 deficient cells treated with Etomoxir had equal survival rate comparable to cells treated with control medium (Figure 2-19).

Higher survival rates of Twist1 deficient once stimulated Th1 cells 3 days after activation reflect importance of glycolysis in activation state and as we have shown, Twist1 downregulates glycolysis. Etomoxir treatment abrogated glycolysis in effector T cells (Figure

2-20a). This indicates that while Twist1 deficiency might affect slightly survival of once stimulated Th1 cells long term, fatty acid oxidation or synthesis does not appear to play a role in a long term survival.

In contrast Etomoxir treatment did not affect glycolysis of repeatedly stimulated Twist WT or deficient Th1 cells (Figure 2-20b). Survival was slightly, but insignificantly affected 3 days after stimulation (Figure 2-19). Nevertheless, the long-term survival was significantly hampered in Twist1 deficient cells on day 7 after activation in both control and fatty acid oxidation limiting condition. However, fatty acid inhibition was affecting Twist1 mediated survival significantly more that control treated cells (Figure 2-19, squared). We did not observe this effect in repeatedly stimulated Th17 cells, suggesting it is Th1 specific (Figure 2-21). We observed higher lipid peroxidation on day 8 after activation in three time stimulated Twist1 deficient cells treated with Etomoxir (Figure 2-18), nevertheless whether it is due to defective protection against lipid peroxides needs to be further determined by employing Ferroptosis (death cause by high levels of peroxidation) inhibition by small molecules, such as Ferrostatin or complementation study, such as overexpression of Gpx4.

4.7 Conclusions and outlook

In this thesis, for the first time, we study the metabolism of rheumatoid Th1 cells. It was shown previously, that naive cells isolated from RA patients do not metabolically meet activation-imposed energy demands as they fail to induce the glycolytic enzyme 6-phosphofructo-2-kinase/ fructose-2,6-bisphosphatase 3 (PFKFB3). This causes accumulation of glucose-6 phosphate (G6P) resulting in increased shuffle of G6P to the pentose phosphate pathway. By action of glucose 6 phosphate dehydrogenase (G6PD) accumulated G6P is converted to ribulose 5 phosphate and NAPDH. As a major factor in ROS scavenging, overproduction of NAPD leads to the downregulation of ROS mediated cell signalling, leading to hyper proliferation of T helper cells (Yang et al. 2016). This study, however, omits influence of stimulation history and synovial environment. Study by Yang et al. goes in line with our findings: NAPDH overproduction might support accumulation of newly formed fatty acids in rheumatic cells. Increased production of lipid intracellular content would give these CD4+ T cells big survival advantage, as oxidation of fatty acids is most efficient energy pathways. Here, we show that *in vitro* repeatedly stimulated murine T cells and human pathogenic T cells isolated *ex vivo* from the site of inflammation of patients suffering from JIA are more reliant on fatty acid oxidation than on glycolysis. Our experiment shows that CD45RO CD4 CXCR5- PD1+, marked as pathogenic cells in inflamed synovium, had higher survival rates than CD45RO CD4 CXCR5- PD1- synovial cells. We also show that

CD45RO CD4 CXCR5- PD1+ cells can be selectively starved *in vitro* by inhibition of fatty acid oxidation. Additionally, we show that CD45RO CD4 CXCR5- PD1+ synovial cells have upregulated *Twist1* that supports *de novo* synthesis of fatty acids and protection against oxygen reactive species. Fatty acids are perfect way to store an energy: triacylglycerol (TAG) can be produced from both carbohydrates and amino acids and be utilized in times during starvation. Is the metabolism of pathogenic cells promising target for treatment? So far, attempts for treatment of Th1 driven chronic inflammation involve blockage of pro-inflammatory cytokines such as TNF-α or IL-6 receptor and lead to amelioration of inflammation (Skurkovich et al. 2001; Ryan et al. 2010). Nevertheless, upon withdrawal of treatment, symptoms relapse. Alternative, symptoms relieving, strategy in patients with rheumatoid arthritis is immunoablation of all $CD4^+$ T cells. Sadly, capacity of immune system to fight against pathogens and opportunistic infections is lowered (Horneff et al. 1991; Brett et al. 1996; Choy et al. ; Emmrich et al.), likely because of depletion efficiency was observed higher in the naive circulating and protective T helper and Treg pool than in the (auto-) antigen experienced memory Th1 cells (Horneff et al.). Complete ablation of immune system followed by autologous stem cell transplantation (ASCT) (Muraro and Abrahamsson ; Alexander et al. ; LoCascio et al. ; Couri et al.) has been another attempt of treatment where it was possible to establish novel immune system with a long-term remission in most patients. However, invasive, complicated nature of the treatment bears risks for patients such as therapy-associated lymphopenia opening window for opportunistic bacterial and fungal infections, development of *de novo* autoimmunity and malignancies in the first years after ASCT (Daikeler et al. ; Holbro et al. ; Marmont ; Couri et al.). Therefore search for novel, non-invasive therapies is still of high interest and our findings on the metabolism of pathogenic immune response hint potential development of new drugs that are able to inhibit selectively metabolic pathways. Utilizing metabolic properties to target pathogenic mechanisms of RA is attractive, in particular since metabolic inhibitors treatment is used in treatment of heart failure, diabetes or cancer (Rupp et al. 2002; Giannessi et al. 2003; Camarda et al. 2016).

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Supplementary Table

Table 4 Genes differentially expressed in repeatedly stimulated Th1 Twist wt/wt versus repeatedly stimulated Th1 Twist fl/fl

The transcriptional profiles of duplicates of cultures were compared using Affymetrix Murine Genome GeneChip arrays. The Affymetrix probe set ID (Affymetr_No),Gene Symbol, Name, Mean Affymetrix Signal, fold change,HPCDA (High Performance Chip Data Analysis) score two arrays per group are shown. Genes were filtered according to the following criteria: fold change >= 1,1; probe signal in wt >= 120; excluding immunoglobulin genes, relational data base for data analysis was created by Joachim Gruen, DRFZ, Berlin).

Affymetrix Number	Gene Symbol	Name	Mean_ Signal Twist $^{wt/wt}$	Mean_ Signal Twist1 $^{fl/fl}$	FC	HPCDA Score
1418733_at	Twist1	twist gene homolog 1 (Drosophila)	1282.25	203.25	5.66	355.47
1423466_at	Ccr7	chemokine (C-C motif) receptor 7	370	126.85	2.78	170.50
1422470_at	Bnip3	BCL2/adenovirus E1B interacting protein 1, NIP3	722.65	439	1.80	177.42
1438239_at	Mid1	midline 1	821.45	539.25	1.74	198.66
1436590_at	Ppp1r3b	protein phosphatase 1, regulatory (inhibitor) subunit 3B	291.6	177.55	1.68	183.82
1420425_at	Prdm1	PR domain containing 1, with ZNF domain	238.6	165.85	1.60	210.20
1423747_a_at	Pdk1	pyruvate dehydrogenase kinase, isoenzyme 1	377.5	226.4	1.57	174.04
1436990_s_at	Ndg2	Nur77 downstream gene 2	626.05	499.85	1.54	192.68
1453556_x_at	Cd99	CD99 antigen	287.4	165.65	1.54	199.00
1454109_a_at	Jmjd6	jumonji domain containing 6	295.3	208.4	1.52	205.34

1418188_a_at	Malat1	Metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	770.8	473.5	1.49	177.49
1417546_at	Il2rb	interleukin 2 receptor, beta chain	323.85	210.35	1.46	188.84
1438504_x_at	Tm7sf3	Transmembrane 7 superfamily member 3	144.7	76	1.46	236.51
1450981_at	Cnn2	calponin 2	466.6	300.7	1.46	190.68
1425927_a_at	Atf5	activating transcription factor 5	317.5	238.1	1.44	173.20
1427532_at	Trat1	T cell receptor associated transmembrane adaptor 1	200.2	138.9	1.44	223.87
1451695_a_at	Gpx4	glutathione peroxidase 4	716.45	516.1	1.44	175.88
1421855_at	Fgl2	fibrinogen-like protein 2	46.55	26.35	1.41	236.28
1422716_a_at	Acp1	acid phosphatase 1, soluble	188.75	125.15	1.41	202.21
1430634_a_at	Pfkp	phosphofructokinase, platelet	147.55	95.9	1.41	219.58
1437052_s_at	Slc2a3	solute carrier family 2 (facilitated glucose transporter), member 3	5269.75	3946.8	1.41	238.34
1450749_a_at	Nr4a2	nuclear receptor subfamily 4, group A, member 2	876.1	680.6	1.37	207.26
1434773_a_at	Slc2a1	solute carrier family 2 (facilitated glucose transporter), member 1	2946.05	2251.1	1.34	191.30
1423423_at	Pdia3	protein disulfide isomerase associated 3	2495.45	1827.6	1.32	228.40
1436750_a_at	Oxct1	3-oxoacid CoA transferase 1	334.35	240.05	1.32	173.19

1453622_s_at	Mllt3	myeloid/lymphoid or mixed lineage-leukemia translocation to 3 homolog (Drosophila)	243.4	171.55	1.32	198.50
1418760_at	Rdh11	retinol dehydrogenase 11	289.05	211.15	1.30	213.23
1423548_s_at	Ergic3	ERGIC and golgi 3	374.95	282.55	1.30	173.43
1416246_a_at	Coro1a	coronin, actin binding protein 1A	2973.65	2341.85	1.27	190.39
1438366_x_at	Clcn3	chloride channel 3	552.3	403.5	1.27	187.50
1450696_at	Psmb9	proteasome (prosome, macropain) subunit, beta type 9 (large multifunctional peptidase 2)	616.25	484.85	1.27	174.70
1455007_s_at	Gpt2	glutamic pyruvate transaminase (alanine aminotransferase) 2	372.85	313.85	1.27	184.56
1422280_at	Gzmk	granzyme K	498.05	369.6	1.25	210.51
1451081_a_at	Tcf25	transcription factor 25 (basic helix-loop-helix)	261	219.45	1.25	189.55
1457120_at	Itk	IL2-inducible T-cell kinase	1120.4	945.7	1.25	177.49
1460469_at	Tnfrsf9	tumor necrosis factor receptor superfamily, member 9	2662.35	2010.95	1.25	204.50
1437249_at	Skap1	src family associated phosphoprotein 1	490.65	378.65	1.23	173.75
1436298_x_at	Paics	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoribosylaminoimidazole, succinocarboxamide synthetase	1748.05	1205.95	1.19	200.49
1436152_a_at	Hbxip	hepatitis B virus x interacting protein	1654.85	1325.8	1.17	229.19
1429005_at	Mfhas1	malignant fibrous	1514	1777.75	-1.21	212.06

		histiocytoma amplified sequence 1				
1437690_x_at	Csnk1d	casein kinase 1, delta	609.4	744.9	-1.21	222.16
1450330_at	Il10	interleukin 10	5822.35	6841.85	-1.21	236.39
1448898_at	Ccl9	chemokine (C-C motif) ligand 9	3208.9	3962.5	-1.23	263.27
1416755_at	Dnajb1	DnaJ (Hsp40) homolog, subfamily B, member 1	560.85	706.3	-1.25	175.52
1421930_at	Icos	inducible T-cell co-stimulator /// similar to activation-inducible lymphocyte immunomediatory molecule AILIM	1275.5	1701.4	-1.25	222.31
1422924_at	Tnfsf9	tumor necrosis factor (ligand) superfamily, member 9	2554.35	3196.1	-1.25	191.17
1434674_at	Lyst	lysosomal trafficking regulator	384.95	499.3	-1.25	197.33
1435557_at	Fhod1	formin homology 2 domain containing 1	149.8	198.55	-1.25	205.96
1451596_a_at	Sphk1	sphingosine kinase 1	980.55	1284.65	-1.25	179.33
1452214_at	Skil	SKI-like	827.65	1168.8	-1.25	179.61
1416083_at	Za20d2	zinc finger, AN1-type domain 5 /// similar to zinc finger protein ZNF216	926.45	1170.3	-1.27	199.95
1416926_at	Trp53inp1	transformation related protein 53 inducible nuclear protein 1	1744.2	2348.8	-1.27	187.59
1420965_a_at	Enc1	ectodermal-neural cortex 1	456.65	583.4	-1.27	183.54
1425745_a_at	Tacc2	transforming, acidic coiled-coil containing protein 2	618.3	772.1	-1.27	175.53
1449235_at	Fasl	Fas ligand (TNF superfamily, member	1406.9	1734.35	-1.27	196.84

		6)				
1452406_x_at	Erdr1	erythroid differentiation regulator 1	2894.05	3552.5	-1.27	212.94
1419208_at	Map3k8	mitogen activated protein kinase kinase kinase 8	1591.9	1913.2	-1.30	181.82
1424090_at	Sdcbp2	syndecan binding protein (syntenin) 2	317.85	423.1	-1.30	180.97
1418926_at	Zeb1	zinc finger E-box binding homeobox 1	391.45	488.25	-1.32	766.63
1420895_at	Tgfbr1	transforming growth factor, beta receptor I	245.25	305.1	-1.32	194.08
1424942_a_at	Myc	myelocytomatosis oncogene	4093.45	5184.6	-1.32	245.99
1426334_a_at	Bcl2l11	BCL2-like 11 (apoptosis facilitator)	173.9	225	-1.34	172.59
1433451_at	Cdk5r1	cyclin-dependent kinase 5, regulatory subunit (p35) 1	333.85	624	-1.34	176.82
1422567_at	Niban	niban protein	1019.15	1364.05	-1.37	876.54
1434129_s_at	Lhfpl2	lipoma HMGIC fusion partner-like 2	105.1	153.9	-1.41	203.12
1450334_at	Il21	interleukin 21 /// similar to interleukin 21	1489.55	2193.65	-1.44	224.51
1456700_x_at	Marcks	myristoylated alanine rich protein kinase C substrate	120.1	222.95	-1.49	173.05
1430001_at	Il31	interleukin 31	290.55	486.05	-1.52	174.96
1427256_at	Vcan	versican	422.35	666.25	-1.62	205.80
1427624_s_at	Il22 /// Iltifb	interleukin 22 /// interleukin 10-related T cell-derived inducible factor beta	103.65	198.85	-2.14	197.69
1417256_at	Mmp13	matrix metallopeptidase 13	146.65	368.7	-2.18	591.79
1426438_at	Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide	123.85	561.15	-17.4	380.52

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Declaration

Hiermit erkläre ich, die Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfen und Hilfsmittel angefertigt zuhaben. Ich habe mich anderwärts nicht um einen Doktorgrad beworben und besitze keinen entsprechenden Doktorgrad. Ich erkläre, dass ich die Dissertation oder Teile davon nicht bereits bei einer anderen wissenschaftlichen Einrichtung eingereicht habe und dass sie dort weder angenommen noch abgelehnt wurde. Ich erkläre die Kenntnisnahme der dem Verfahren zugrundeliegenden Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt-Universität zu Berlin vom 6. Juli 2009. Weiterhin erkläre ich, dass keine Zusammenarbeit mit gewerblichen Promotionsberaterinnen/Promotionsberatern stattgefunden hat und dass die Grundsätze der Humboldt-Universität zu Berlin zur Sicherung guter wissenschaftlicher Praxis eingehalten wurden.

Berlin, September, 2017

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